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UNIVERSITY OF ALBERTA

Poxvirus DNA Replication and the Resolution of Telomere Replicative Intermediates

BY

© David Stuart

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF Biochemistry

Edmonton, Alberta
SPRING 1992

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Happy new year to you, Grant, and all the rest of the lab.

Yours,

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"It's hard to make a good theory-a theory has to be reasonable, but the facts do not"

George, W. Beadle

"One can spend too much clean thought on clean proteins, if one does not have all of the parts"

Bruce, M. Alberts

Pereant qui ante nos nostra dixerunt

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ABSTRACT

The linear DNA genomes of poxviruses provide a paradigm for the replication of hairpin terminated chromosomes. The replication of poxvirus DNA results in the formation of high molecular weight concatemeric arrays of nascent viral genomes linked end-to-end by fusions of the telomere sequences. During the process of maturation the telomere fusions are resolved into progeny hairpin telomeres by a site-specific recombination event that is dependent upon a 20 nucleotide *cis*-acting telomere resolution target sequence (TRT).

Poxvirus telomere resolution has been investigated by subjecting a cloned version of the Shope fibroma virus (SFV) replicative intermediate to site-specific mutagenesis. Efficient resolution *in vivo* requires that the replicative intermediate structure retain perfect or near perfect inverted repeat symmetry. The cis-acting TRT sequence required for telomere resolution is required in two copies, inverted with respect to one another, and in a specific orientation with respect to the axis of symmetry. Correctly oriented TRTs confer *in vivo* resolution to an inverted repeat structure in a distance dependent fashion. That is if the two sequences are placed beyond a minimum distance apart they no longer function as resolution targets.

It has been determined that the TRT sequence serves a dual role *in vivo*, it functions not only as a target sequence to confer resolution upon replicative intermediate structures but also serves as a strong viral specific late promoter. The TRT sequence does not regulate the expression of any open reading frames but its ability to act as a promoter is tightly correlated with its ability to act as a resolution target. The transcription and resolution functions of the TRT sequence can not be unlinked either by mutating the promoter or by the use of inhibitors of transcription. Extracts derived from poxvirus infected cells that are capable of initiating RNA synthesis from poxvirus late promoters are able to resolve cloned replicative intermediate structures *in vitro*. It is argued based upon this evidence that the viral transcription complex or an associated subunit has a specific role in telomere

resolution. The TRT sequence may thus act to target the resolution proteins and transcriptional complex to the telomere replicative intermediates.

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List of Terms and Abbreviations

Amp^r	ampicillin resistant
Ara C	cytosine-β-D-arabinofuranoside
ATP	adenosine triphosphate
bp	nucleotide basepair
CAT	chloramphenicol acetyl transferase
dATP	2' deoxyadenosine 5' triphosphate
dCTP	2' deoxycytidine 5' triphosphate
dGTP	2' deoxyguanosine 5' triphosphate
dTTP	2' deoxythymidine 5' triphosphate
dUTPase	deoxyuridine triphosphate nucleotidohydrolase
dsDNA	double stranded DNA
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ffu	focus forming unit
Kan^r	kanamycin resistant
kbp	kilobasepair
kDa	kilodalton
Me$_2$SO	dimethyl sulfoxide
MOI	multiplicity of infection
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NP40	a nonionic detergent
ORF	open reading frame
pfu	plaque forming unit
rDNA	ribosomal DNA
SFV	Shope fibroma virus
ssDNA	single stranded DNA
Tet^r	tetracycline resistant
T. K.	thymidine kinase
Tris-HCl	tris(hydroxymethyl) amino methane hydrochloride
transfection	The introduction of DNA into a recipient eukaryotic cell.The procedures in this thesis all refer to DNA transfer mediated by calcium

	phosphate.
transformation	Any alteration in the properties of a cell that are stably inherited by its progeny. In this thesis, transformation refers to the plasmid mediated aquisition of drug resistance by bacterial cells or the changes caused in cultured eukaryotic cells by tumor viruses.
ts	temperature-sensitive
UNG	uracil DNA glycosylase

Chapter I

Introduction

DNA replication stands among the most universal and most elegant of biological processes. At the simplest level DNA synthesis requires the unwinding of duplex DNA and the copying of each parental strand in a template directed fashion by a DNA dependent DNA polymerase. This relatively simple mechanistic process is the way in which DNA replication is generally considered and at this level the process does indeed approach universality. Stepping up one level from DNA synthesis to the replication of chromosomes, a remarkable diversity becomes evident with regard to chromosome structure and the processes of replication initiation, priming, elongation, termination, and resolution of replicative intermediates. This diversity provides a very clear example of how a biological problem can be solved in many different ways.

The subject of this thesis is DNA replication, with emphasis on the resolution of replicative intermediate DNA structures and the segregation of nascent daughter chromosomes. These studies were undertaken using the poxvirus, Shope fibroma virus (SFV), as a model system. Poxviruses provide a unique model for the study of DNA replication for the following reasons. (i) Poxvirus replication occurs in the cell cytoplasm and while some aspects of poxvirus biology are typically eukaryotic, a complete and distinctive "replication machine" is encoded by the viral genome. A significant advantage of the relatively compact viral genome is that the encoded replicative machinery is susceptible to genetic analysis and manipulation. (ii) Poxviral chromosomes have the interesting although not novel feature of hairpin termini and present an excellent model for the study of the replication of hairpin termini.(iii) Poxviruses provide a useful model to determine the mechanism by which the intermediate structures resulting from the replication of hairpin termini are resolved into progeny hairpin termini.

The resolution of DNA replicative intermediate structures is a crucial final event in the process of DNA replication that has prompted the evolution of gene products that function (sometimes uniquely) to perform specific activities related to the resolution of the intermediate structures generated by chromosome replication.

Observations of the nature of poxvirus replicative intermediate structures and the mechanism of their resolution within an integrated framework of nucleic acid metabolism form the basis of this thesis.

A. PHYSICAL CHARACTERISTICS OF POXVIRUSES

The physical characteristics of poxvirus virions have been reviewed in detail (Dales & Pogo, 1981). Based upon electron microscopic (E. M.) observation, most members of the chordopoxviridae are very similar in appearance. The purified intracellular form of the vaccinia virus particle was originally referred to as an elementary body (E.B.) (Bland & Robinson, 1939). Initial observations of dehydrated E. B.s indicated that the particles are oval or brick shaped with dense internal structures. Some of the internal subvirion components were initially visualized under the E. M. by controlled protease digestion and heavy metal shadowing (Peters & Meuller, 1963). This type of analysis provided the first description of the dense core or nucleoid with its thick protein coat enclosing the tightly coiled viral genome complexed in a nucleoprotein structure (Peters & Meuller, 1963). The outer surface of the core is covered with protruding structures that emanate from the protein coat. Large proteinaceous masses of unknown function called lateral bodies occupy the space between the core and the envelope. Under E. M. analysis, thin sectioned virions display a lipoprotein bilayer (outer membrane) surrounding the core nucleoid and lateral bodies; protruding from this core are numerous external cylindrical structures called surface tubular elements (STE) (Dales, 1962). Vaccinia virus is typical of the Orthopoxviruses and displays a length-width ratio of approximately 1.3. The virion length varies from 235-280 nm and its width from 165-225 nm; much of the variability is likely due to dehydration and other sample preparation procedures. Early electron microscopic analysis of the structure of the Leporipoxviruses SFV and Myxoma indicate that at the gross level they are very similar to the Orthopoxvirus, vaccinia virus (Lloyd & Kahler, 1955).

During ultracentrifugation the vaccinia virus particle sediments at 4910 Svedberg units indicative of its large size and globular structure. The dry weight has been estimated to be 5.5×10^{-15} grams/E. B. (Smadel & Hoagland, 1942). The large size of poxvirus virions and relative ease with which they can be purified via differential centrifugation led to an early chemical characterization of the virion components. Orthopoxvirus virions are composed of approximately 90% protein, 5% lipid and 4% DNA but no RNA (Smadel & Hoagland, 1942). The particular lipid composition of the envelope varies with the virus strain and the host cells in which the virus is produced. The viral membrane consists of phospholipid, cholesterol and some glycolipid components. Stripping off the lipid bilayer decreases infectivity; however, the mechanism is unclear (Zwartouw, 1964). The composition of the viral lipid envelope differs from the composition of host cell membranes (Dales & Mosbach, 1968), but it is unlikely that the virus encodes enzymes for lipid biosynthesis. Differences in membrane composition suggest that poxviruses are capable of selectively directing the incorporation of specific host phospholipid and glycolipid species into their own membranes. Extracellular forms of the virus are wrapped in a cellular plasma membrane in addition to the *de novo* synthesized viral lipid bilayer (Payne & Norrby, 1976). The significance of the cellular wrapping is unclear but it likely functions in some way to enhance infectivity.

The large vaccinia virus virion is a complex of over 100 polypeptides that can be visualized by two-dimensional gel-electrophoresis (Essani & Dales, 1979). The membranes of extracellular forms of the vaccinia virus contain at least eight polypeptides, seven of which are glycosylated (Payne, 1978). The outer membrane of the intracellular form of vaccinia virus contains five polypeptide species that can be surface labelled with I^{125} (Sarov & Joklik, 1972) and can be stripped off with the non-ionic detergent NP-40. Four major proteins of 74, 62, 25 and 11 kDa compose 70% by weight of the vaccinia virus core. The first three of these are derived by proteolytic processing of a high molecular weight precursor.

The genomes of poxviruses consist of a single, long double-stranded DNA molecule of approximately 160-170 x 10^6 Da for vaccinia virus (Joklik, 1962) and 153 x 10^6 Da for SFV (Jacqemont *et al.*, 1972). The Copenhagen strain of vaccinia virus has been entirely sequenced and is composed of 191,636 nucleotide pairs corresponding to a molecular weight of 125 x 10^6 Da (Goebel *et al.*, 1990).

The most detailed studies of poxvirus genomes have been carried out with the Orthopoxvirus vaccinia virus. Restriction maps of different strains of vaccinia as well as some other closely related Orthopoxviruses are very similar with most of the differences residing in the terminal 10 to 20 kbs of DNA. Fine restriction mapping of the termini of vaccinia virus revealed a very complex arrangement of short repetitive sequences (Wittek & Moss, 1980). The number of repeated sequences and their arrangement varies among virus isolates, but in one typical example (see Fig. I-1), the first repetitive element of 70 bp starts at 87 bp from the terminus and is repeated 13 times followed by a unique 325 bp sequence, and then 18 more repeats of the 70 bp sequence. The last repeat is immediately followed by 2 tandem 125 bp repeats and then 8 more 54 bp repeats (Wittek & Moss, 1980). All of the well characterized Orthopoxviruses have some similar version of the terminal tandem repeat arrangement (Pickup *et al.*, 1982, Parsons & Pickup, 1987). The function of the noncoding repetitive sequences is unknown, however, it is believed that the variability among isolates is due to unequal intermolecular crossover events (Baroudy & Moss, 1982). The Leporipoxviruses do not have tandem repeats, but do have a series of imperfect inverted repeat sequences near the termini (DeLange *et al.*, 1986). Another interesting feature of the genomes of vaccinia virus (Garon *et al.*, 1978) and SFV (DeLange *et al.*, 1984b) is that they consist of sequences that are identical at either end of the genome but are oppositely oriented, that is the ends of the genome consist of terminal inverted repeats (TIRs) (Fig. I-1). Thus, any genes encoded within the TIRs are present in two copies per genome. The TIR of SFV encodes a series of 9 tandemly arranged genes all of which are transcribed toward the terminus (Upton *et al.*, 1987, Macaulay *et al.*, 1987). The TIR

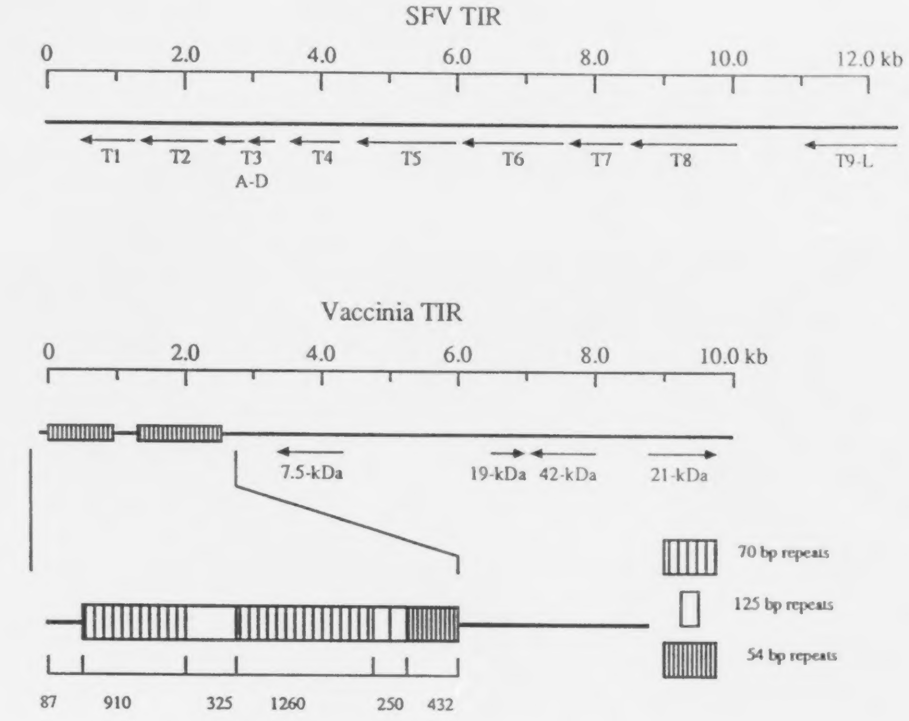
SFV TIR
0
2.0
4.0
6.0
8.0
10.0
12.0 kb
T1
T2
T3
A-D
T4
T5
T6
T7
T8
T9-L
Vaccinia TIR
0
2.0
4.0
6.0
8.0
10.0 kb
7.5-kDa
19-kDa
42-kDa
21-kDa
70 bp repeats
125 bp repeats
54 bp repeats
87
910
325
1260
250
432

Figure I-1. A schematic representation comparing the TIR regions in the linear genomes of the leporipoxvirus SFV and the prototypical orthopoxvirus vaccinia virus. The length of the regions is indicated in kilobases (kb) above each TIR representation. The arrows indicate open reading frames in the TIR of each virus and the direction in which each gene is transcribed. The repeated sequence elements that compose the terminus of vaccinia virus are indicated by the blocks at the extreme left of the vaccinia virus TIR.

region of vaccinia virus encodes four genes; two are transcribed toward the termini and two away from the termini (Wittek *et al.*, 1980a,1980b).

Intact viral DNA purified from virions displays the ability to rapidly reanneal following denaturation (Berns & Silverman, 1970). The rapid reannealing or "snapping back" is due to covalent phosphodiester linkages at the extreme termini of the genomes that result in cross linking of the termini so that the entire genome exists as one entirely closed self-complementary molecule (Baroudy *et al.*, 1982, Geshelin & Berns, 1974). The covalently closed hairpin nature of the telomeres was verified by sequencing the termini of vaccinia virus (Baroudy *et al.*, 1982) and SFV (DeLange *et al.*, 1986). Sequencing the viral telomeres indicated not only that the termini exist as covalently closed hairpin structures, but also that unpaired, extrahelical bases are present in the hairpin region; 8 in the case of SFV and 12 in the case of vaccinia virus. The significance of the unpaired bases is unknown but they are a conserved feature that causes the genomic termini of SFV and vaccinia virus to adopt two different isomeric forms, "flip" and "flop", that are distinguishable by their sequences and their differing mobility during electrophoresis through native polyacrylamide gels (Baroudy *et al.*, 1982, 1983).

B. POXVIRUS REPLICATIVE CYCLE

(i)Virus Entry.

Poxviruses gain entry into host cells through an apparently nonspecific membrane fusion followed by active phagocytosis. The initial adsorption stage is very rapid and is not dependent on any active process by either the virus or the host cells. Even heat killed virus will adsorb to cells and virus adsorbs to either live or dead cells with similar efficiency (Febvre, 1962, Dales & Pogo, 1981). Virus particles fused to membranes within vacuoles formed by surface invagination (Dales and Kajioka, 1964) and also fused to the plasma membrane at the surface of cells (Chang & Metz, 1976) have been observed during electron microscope investigations of viral invasion. Fusion with the plasma membrane most likely occurs in a pH independent fashion because lysosomotropic agents do not inhibit plaque formation (Janeczko *et al.*, 1987). The presence of a specific cellular receptor for poxvirus entry can not be discounted; however, it seems unlikely given the extremely broad range of cells that can be entered by vaccinia virus and SFV. The EGF receptor has been suggested as a possible receptor for vaccinia virus (Eppstein *et al.*, 1985), but this seems unlikely due to the inability of EGF to entirely compete out virus entry (Eppstein *et al.*, 1985) and the ability of vaccinia to infect cells that do not express EGF receptors (Stroobant *et al.*, 1985). Some of the viral envelope proteins may have a role in the fusion with cell membranes. Antibodies against five virion proteins can neutralize infectivity (Oie & Ichihashi, 1978, Rodriquez *et al.*, 1987, Stern & Dales, 1976). A 14 kDa protein has been implicated in membrane fusion and mutations in this protein result in a decreased plaque size (Dallo *et al.*, 1987, Rodriquez *et al.*, 1987). Following adsorption the virus is taken up via an active phagocytic process that is in some way directed by viral envelope proteins. Heat killed viruses adsorb to cells and are taken up; however, they are shunted to lysosomal vesicles where they are completely dismembered and destroyed, while only a very small portion of live infective virus is directed to lysosomes (Dales & Pogo, 1981). The mechanism by which the poxvirus directs its own entry into the host cell is unknown but presumably some virion component facilitates entry directly into the cytoplasm and prevents its being targeted to lysosomes.

(ii) Viral Uncoating.

The uncoating of poxvirus DNA is a two step process (reviewed in Dales & Pogo, 1981). E. M. photos indicate that following interaction with cell membranes and phagocytic engulfment, viral cores are released into the cytoplasm with the lateral bodies still loosely attached. This initial stage of uncoating can be monitored biochemically by a change in the particle sedimentation rate and by solubilization of viral phospholipid and some viral membrane proteins. The phospholipid becomes associated with the cellular membranes while the envelope proteins are degraded (Joklik, 1964a). This first phase of uncoating (phase I uncoating) happens very rapidly and is independent of any new RNA or protein synthesis. Even heat killed virus can be uncoated to this extent. The second phase of uncoating (phase II uncoating) follows a lag that is dependent upon the multiplicity of infection (Joklik, 1964b). During this uncoating stage the lateral bodies break away from the core and are no longer visible by E. M., the core then ruptures to release the viral genome and encapsidated enzymes into the cytoplasm. The rupture of cores can be assayed biochemically by the sensitivity of the viral DNA to DNase I. The second phase of uncoating requires the synthesis of new viral proteins (Joklik, 1964b). In the presence of inhibitors of protein synthesis, the infecting viral cores accumulate in the host cell cytoplasm. Once uncoating activity is activated in the cytoplasm there is no lag phase for the uncoating of superinfecting virions of the same or a heterologous poxvirus (Joklik, 1964b). Evidence that the uncoating activity is virally encoded comes from the infection of cells pretreated with actinomycin D (Magee & Miller, 1969) and from the ability of vaccinia virus to uncoat and synthesize DNA in enucleated cells (Prescott *et al.*, 1971, Pennington & Follet, 1974, Hruby *et al.*, 1979). In both cases the viral genomes are uncoated following a characteristic lag phase. An uncoating activity has been partially purified from poxvirus infected cells (Pedley & Cooper, 1987). The trypsin like enzyme will uncoat the viral cores of vaccinia or cowpox but fails to uncoat enveloped virus . No gene has yet been assigned to the uncoating activity.

(iii) Early Gene Expression

The poxvirus virion encapsidates all of the viral gene products required for the transcription of the early class of viral genes. These include the viral RNA polymerase which is distinct from the cellular RNA polymerase II in its subunit composition and alpha-amanitin resistance (Nevins & Joklik 1977). The viral RNA polymerase is a multisubunit enzyme consisting of two large polypeptides and numerous smaller polypeptides (Baroudy

& Moss, 1980). The genes for the 147,132, 35, 30, 22, 19, and 18 kDa subunits have been identified and characterized (reviewed by Moss, 1990a, 1990b). The early transcription factor (VETF), is a DNA dependent ATPase, composed of two subunits of 82 kDa and 77 kDa. VETF directs promoter specific transcription activity by binding to and bending early promoter sequences (Broyles *et al.*, 1991, Broyles, 1991). The mRNA "capping" reactions of guanylyl-transfer and methylation are carried out by a virus encoded 127 kDa multifunctional enzyme complex composed of 97 and 33 kDa subunits (Martin *et al.*, 1975, Shuman & Hurwitz, 1981). The genes for the large and small subunits of the vaccinia virus capping enzyme have been identified (Morgan *et al.*, 1984 , Niles *et al.*, 1989), and the gene encoding the large subunit of the SFV enzyme has been identified (Upton *et al.*, 1991). The 3' polyadenylation of early transcripts is performed by the encapsidated poly (A) polymerase. This enzyme is composed of two subunits with masses of 55 kDa and 33 kDa. The genes encoding the subunits of the vaccinia virus version of this enzyme have recently been identified and sequenced (Gershon *et al.*, 1991). There has been some suggestion that host RNA polymerase subunits are involved in transcription of the poxvirus genome (Morrison & Moyer, 1986). However, the synthesis of viral RNA in enucleated cells suggests that this is probably not the case or at least is not an essential feature of the viral replicative cycle.

Three distinct classes of poxvirus genes are expressed in a cascade pattern (Fig. I-2). Each temporally distinct class of viral gene can be distinguished by the presence of signature promoter sequences 5' to the coding sequence. The early genes are expressed prior to the initiation of DNA replication and the products of these genes include enzymes required for the biosynthesis of DNA precursors as well as the enzymes required for DNA synthesis. Newly synthesized viral RNA can be detected shortly after the first stage of uncoating and is required for the second stage of uncoating to occur (Kates & McAuslan, 1967). Indeed purified virions that have been "uncoated" with NP-40 are capable of synthesizing *bona-fide* early RNA if incubated in the presence of the ribonucleotide triphosphate precursors of RNA (Kates & McAuslan, 1967). The *in vitro* synthesized RNA accumulates in the virion and requires a high concentration of ATP in order to be extruded (Kates & Beeson, 1970, Shuman *et al.*, 1980). This not only demonstrates the presence of the transcriptional machinery in the virion, it also indicates that the encapsidated enzymes are loaded on to the viral DNA in such a fashion that they are capable of immediately synthesizing mRNA. The synthesis of early RNA is enhanced when the infected cells are treated with protein synthesis inhibitors that prevent phase II uncoating of the viral cores (Woodson, 1967). In this case the viral genome and transcriptional enzymes are protected from the cellular *milieu* and so early gene expression can continue unabated. The synthesis of early RNA is somewhat prolonged when the infected cells are treated with inhibitors of viral DNA replication. In this case the viral DNA and transcriptional apparatus are exposed to the cellular enzymes and the synthesis of early RNA will slowly decrease and eventually cease likely due to the effects of dilution and turnover of the viral proteins by cellular enzymes (Woodson, 1967, Moss, 1990b). Thus the transcriptional apparatus can be maintained in a functional and stable fashion within the virion. Reporter genes that are regulated by viral early gene promoter sequences are poorly expressed when transfected into poxvirus infected cells (Cochran *et al.*, 1985). This observation may be explained by the fact that much of the early transcription occurs within the viral core which is inaccessible to the transfected DNA.

The early RNA synthesized by vaccinia virus is characteristically of discrete length and contains no intervening sequences that require splicing (Cooper *et al.*, 1981a). The mRNA is modified by the addition of a methylated "cap" structure by the viral mRNA capping enzyme (Boone & Moss, 1977, Martin & Moss, 1975) and is also 3' polyadenylated (Kates & Beeson, 1970, Brakel & Kates, 1974a, 1974b). Poly (A) addition is most likely carried out by the viral poly (A) polymerase because the viral RNA lacks the cellular polyadenylation signal. The *cis*-acting signals that regulate poxvirus early transcription are short and distinct from the host promoter sequences (Ink & Pickup, 1989). The consensus early viral promoter consists of a tripartite signal composed of a 16 bp critical region, an 11 bp spacer region and a 7 bp initiation region (Davison & Moss, 1990). A similar promoter structure has been defined for the Leporipoxvirus SFV and it has been shown that the Orthopox and Leporipox viruses will utilize each others early promoter sequences (Macaulay & McFadden, 1989). The discrete nature of poxvirus early transcripts is due to a specific termination signal in the form of the oligonucleotide sequence TTTTTNT (Yeun & Moss, 1987). This signal is located 20-50 bp upstream of the 3' end of most vaccinia virus and SFV early genes. This oligonucleotide sequence is necessary and sufficient for the termination of early transcripts. The presence of the termination signal within the sequence of a foreign gene can result in its premature termination when being transcribed by the viral transcriptional apparatus indicating that no other viral sequence is required to effect the termination of early RNA. It has been observed, however, that in some circumstances when the early transcription termination signal appears within an early gene, it is ignored or "read-through" (Earl *et al.*, 1986). This may be due to the secondary structure of the transcript in this region (Luo & Shuman, 1991). An *in vitro* termination assay has made it possible to determine that the signal for termination lies in the UUUUUNU sequence of the transcribed mRNA rather than the template DNA since termination is inhibited by halogenated uridine residues (Shuman & Moss, 1988). It has recently been demonstrated in a rather definitive fashion *in vitro* that the vaccinia virus mRNA capping enzyme complex functions as the early termination factor (Luo *et al.*, 1991).

(iv) DNA Replication.

Vaccinia virus DNA synthesis can be detected starting about 2 hours post-infection (Fig. I-2). Based on the incorporation of [^{3}H]-thymidine, DNA synthesis reaches a peak at about 3 hours post-infection and from there rapidly declines to a very low level after about 5 hours (Joklik & Becker, 1964). The initiation of DNA synthesis requires that the viral core be uncoated (phase II uncoating) and that early transcription and protein synthesis be allowed to proceed (Esteban & Holowczak, 1978). Prior to DNA replication a series of enzymes required for the synthesis of deoxyribonucleoside precursors is induced, including thymidine kinase, thymidylate kinase and ribonucleotide reductase. A gene with homology to herpes virus dUTPases has been identified in the vaccinia virus genome (McGeoch, 1990); however, its activity remains to be verified. This array of biosynthetic enzymes causes a rapid change in the ribonucleotide and deoxyribonucleotide precursor pools within an infected cell (Slabaugh *et al.*, 1991) and may in part explain the inability to detect ongoing DNA replication by [^{3}H]-thymidine incorporation after 5 hours even when Southern hybridization indicates that viral DNA continues to accumulate for up to 10 hours post-infection (Rempel *et al.*, 1990). Another factor leading to the inability to label replicating viral DNA with nucleoside precursors is the severely decreased ability of the host cell to transport thymidine at late times during the vaccinia virus infection (see appendix A). The synthesis of viral DNA polymerase heralds the onset of DNA replication in infected cells. Poxvirus DNA replication occurs in cytoplasmic foci referred to as virosomes or "factories" (Cairns, 1960). Although these structures do not appear to be membrane bound, the DNA-protein aggregate is sufficiently stable to survive isolation by sucrose gradient sedimentation (Dahl & Kates, 1970). The details of DNA replication and the viral proteins required will be discussed in section (C).

(v) Intermediate and Late Transcription

The replicative cycle of poxviruses is sequential and dependent; that is, each new stage in the cycle is dependent upon the completion or initiation of a previous stage (Fig. I-2). Early transcription leads the the accumulation of enzymes required for DNA replication and at least two gene products that are required as auxiliary factors for the expression of an intermediate class of gene products (Vos & Stunnenberg, 1988, Vos *et al.*, 1991a). Intermediate genes are not expressed in the absence of DNA replication even though RNA polymerase and intermediate transcription factors are present. However, if naked viral DNA or a plasmid borne gene regulated by an intermediate class promoter sequence is

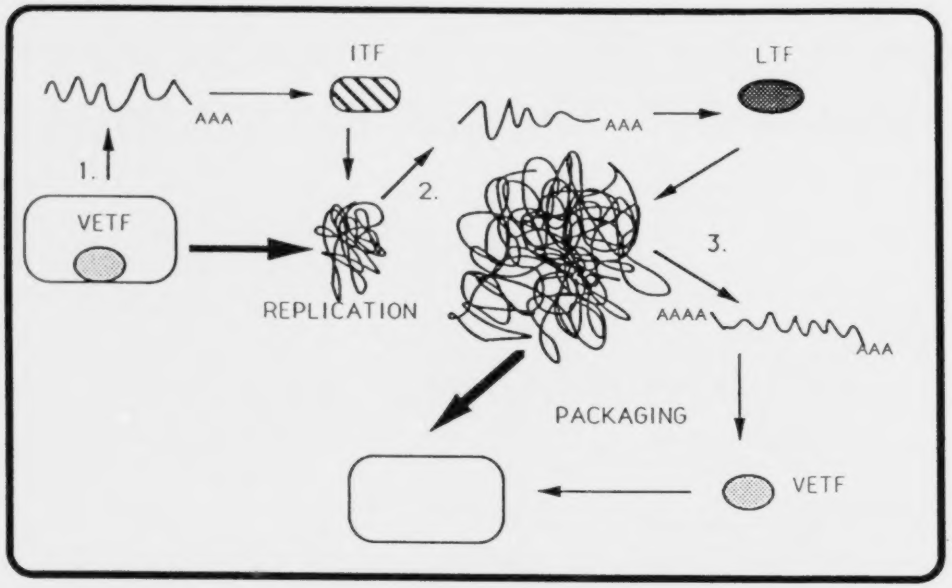

Figure I-2. Cascade regulation of vaccinia virus gene expression. (1.) Early transcription is directed by the vaccinia virus early transcription factor (VETF) in cores. The early genes encode functions required for DNA replication and intermediate transcription factors (ITFs). (2.) ITFs interact with the newly replicated naked genomic DNA initiating intermediate gene expression. Some of the intermediate genes code for late transcription factors (LTFs). (3.) LTFs interact with replicating DNA and the RNA polymerase to direct the expression of late genes. Many of the late genes encode virion structural proteins or enzymes that get packaged and are required for the expression of early genes, among these are the two subunits of VETF, mRNA capping enzyme and RNA polymerase subunits. (Adapted from Traktman, 1990).

transfected into an infected cell that is blocked in DNA replication, the transfected genes will be transcribed while the genomic copy remains unexpressed (Vos & Stunnenberg, 1988). These data suggest that the replication of the incoming viral DNA provides copies of naked template for the intermediate transcription factors to bind to and activate transcription. One of the intermediate transcription factors, VITF B has been shown to be required for the formation of a stable preinitiation complex on intermediate class promoters. VITF B factor alone, in the absence of RNA polymerase can cause ATP dependent unwinding of the DNA in the intermediate gene promoters (Vos *et al.*, 1991a). Addition of the purified intermediate transcription factors to an early specific virion derived transcription extract renders the early RNA polymerase capable of transcribing intermediate genes (Vos *et al.*, 1991a). Unexpectedly, the second intermediate transcription factor, VITF A has been shown to be the mRNA capping enzyme (Vos *et al.*, 1991b). Three of the recently identified intermediate genes that are expressed under the direction of the intermediate transcription factors encode factors that are required for the expression of late gene products (Keck *et al.*, 1990). One of these factors VLTF A has been purified (Wright *et al.*, 1991) and conditional lethal mutations in this gene render the virus unable to synthesize late gene products at the non-permissive temperature (M. Carpenter personal communication). The intermediate genes are not yet well characterized and a detailed description of the RNA synthesized from them is not available.

Following the initiation of DNA replication a switch occurs in transcription such that the promoters for early genes are no longer recognized and first intermediate and then late genes are expressed (reviewed by Moss, 1990b). The molecular mechanism that governs this switching mechanism has not yet been elucidated; however, one simple explanation is that the newly synthesized RNA polymerase subunits associate with the nascent transcription factors that are expressed in excess and titrate out the polypeptides that were originally present in the virion. This can explain the coordinated activation and shut off of the vaccinia virus DNA polymerase gene even when DNA replication and viral late protein synthesis are prevented (Traktman, 1990). The possibility that late proteins repress the transcription of early genes can not be formally excluded since the expression of late genes is required to observe the shutoff of some early genes (McAuslan, 1963). However, transcriptionally competent extracts derived from infected cells at late times are capable of initiating transcription from both early and late promoters (Foglesong, 1985). Inhibition of late gene expression allows the prolonged synthesis of early gene products whose mRNA is relatively stable with a half life of approximately 5 hours (Oda & Joklik, 1967, Sebring & Salzman, 1967).

RNA synthesized at late times during infection is fundamentally different from RNA that is synthesized prior to DNA replication. DNA-RNA hybridization experiments have demonstrated that all or nearly all of the genome is transcribed at late times (Paoletti & Grady, 1977). The RNA synthesized from late promoters has no defined 3' end and the RNA initiated at one late gene promoter can extend through several downstream genes to generate a polycistronic message (Cooper *et al.*, 1981). One result of this heterogeneous pattern of RNA synthesis is that polycistronic messages synthesized from the complementary DNA strands can anneal into RNase resistant RNA-RNA duplexes at late times during the infection (Boone *et al.*, 1979). Yet another peculiar but not entirely novel feature of poxvirus late RNA is the presence of a long stretch of up to 30 polyadenylate residues added to the 5' end of the transcript (Bertholet *et al.*, 1987, Schwer *et al.*, 1987). The polyadenylate leader is the result of pseudo-templated transcription (Jacques & Kolakofsky, 1991), a situation where the RNA polymerase slips or stutters over the repeated T residues in the ATTTA motif (Schwer & Stunnenberg, 1988, Ink & Pickup, 1990) that is a mandatory component of late gene promoters (Davison & Moss, 1990). When the ATTTA motif is incorporated into the start site of an early gene, the RNA synthesized from this promoter at early times has a non-template directed polyadenylate leader (Ink & Pickup, 1990). Some early genes have been identified in vaccinia virus that naturally possess ATTTA sequences at the transcription initiation site and these genes all possess polyadenylate leader sequences (Ink & Pickup, 1990). The function of the polyadenylate leader sequence is not known; however, one function may be to allow the interaction with ribosomal subunits and so allow the initiation of translation even though the body of the RNA is hybridized in a complementary RNA-RNA duplex. This could effectively inhibit the translation of early RNA, which does not possess a non-templated polyadenylate leader, while allowing the translation of the late RNA (Moss, 1990b).

(vi) Viral Morphogenesis

The first virion components that become visible by E. M. in the cytoplasmic virosome are the *de novo* synthesized membrane envelopes that appear as crescent shaped cupulae (Dales & Pogo, 1981). The lipid bilayer is generated using host cell phospholipid and cholesterol acquired from both pre-existing and newly synthesized molecules (Stern & Dales, 1974). The differing phospholipid composition from the host membranes implies that viral proteins may direct the incorporation of cellular phospholipids possibly through the use of phospholipid exchange proteins (Stern & Dales, 1974). The bilayer membrane has a brush like border of protein spicules on the convex surface and granular material adjacent to the concave surface. The spicules form the backing for the membranes and without spicules the membranes loose their characteristic crescent shape (Dales & Mosbach, 1968). The spicule backed membranes are a product of early functions and the envelopment of maturing viral particles by the de novo synthesized bilayer membrane is dependent upon transcription at 2-2.5 hours and translation at 3-3.5 hours. The envelopes are assembled sequentially into uniform spherical particles that enclose viral DNA and an array of early and late enzymes. Only 30-50 % of the nascent viral DNA is packaged into mature virions (Joklik & Becker, 1964). The process of packaging remains obscure and it is not clear whether the membranes assemble around a preformed array of DNA and enzymes or if these are injected following the synthesis of immature virus particles. Some stages of the assembly process can be disrupted by inhibitor drugs. In the presence of some metabolic inhibitors, immature viral particles can be enveloped by a lipid bilayer before the viral DNA has been packaged (Morgan, 1976, Pogo & Dales, 1971). The next step in maturation is the loss of the surface spicules with a concomitant change in shape from spherical to the characteristic brick shaped virion morphology. This step is accompanied by a change in the internal structures from the granular viroplasm to a more structured core with associated lateral bodies. The internal differentiation of the poxvirus virion is dependent upon late functions which include proteolytic processing of precursor proteins (Stern *et al.*, 1977). Inhibition of transcription or translation at late times has no effect upon the formation of immature particles but prevents the assembly of mature virions (Dales & Mosbach, 1968). The final viral proteins to be incorporated into the maturing virion are the surface tubular elements that replace the spicules (Essani *et al.*, 1982). The mature vaccinia virus particles are wrapped in golgi membranes and transported to the periphery of the cell (Ichihashi *et al.*, 1971). This double layered membrane can fuse with the plasma membrane leading to budding and externalization of the nascent virion (Payne & Kristensson, 1979). Only a portion (1-30%) of the total virus produced becomes extracellular; this proportion is dependent on the strain of virus and the infected cell type (Payne, 1979).

C. Replication of Poxvirus DNA

(i) Timing and Localization of DNA Replication

Following synchronous infection, poxvirus virions enter the host cell and undergo the initial phase of uncoating. Early transcription initiates immediately and a pool of viral gene products accumulates in the vicinity of the viral core. Rupture of the core by virally induced activities is required for the initiation of viral DNA replication. Synthesis of viral DNA can initially be detected 1-2 hours post-infection dependent upon the multiplicity of infection and the cell type infected (Joklik & Becker, 1964). At higher multiplicities of infection DNA synthesis is initiated earlier possibly due to the more rapid uncoating of the viral genome. In any infected cell DNA synthesis may initiate synchronously in one or more discrete cytoplasmic foci (Dales & Pogo, 1981). The synthesis of viral DNA is dependent upon early gene products (Joklik & Becker, 1964) and takes place exclusively in the cytoplasm of the host cell (Cairns, 1960, Hruby *et al.*, 1979). The cytoplasmic site of DNA synthesis demands that poxviruses either encode or induce all of the required DNA synthetic enzymes. Transport of poxvirus DNA into the nucleus of host cells has been reported (LaColla & Weissbach, 1975). The significance of these reports is unclear but it is likely that the observations were principally due to the inability to quantitatively exclude virosomal DNA from the nuclear subfractions. Indeed, the complete independence of poxvirus DNA replication from any cellular function is indicated by the ability of enucleated cells or cells pre-treated with inhibitors of RNA synthesis to support viral DNA synthesis (Hruby *et al.*, 1979). The synthesis of viral DNA reaches a peak at 2-4 hours and then appears to be abruptly shut off by 5 hours. During this five hour period a total volume of DNA equivalent to approximately 30-50% of the cellular DNA content is synthesized (Joklik & Becker, 1974). Ongoing protein synthesis is required for continued DNA synthesis and experimental inhibition of protein synthesis allows the completion of the round of replication in progress but prevents reinitiation events (Esteban & Holowczak, 1978). This observation suggests either that some part of the replication machinery is inherently unstable or that each functional replication complex is dependent upon assembly in the presence of nascent subunits.

The above data for the timing of viral DNA replication were obtained through the typical method of measuring incorporation of [^{3}H]-thymidine into newly synthesized DNA. Hybridization of a saturating amount of labelled vaccinia virus DNA to the DNA obtained from equal numbers of infected cells, indicates that viral DNA continues to accumulate at a constant rate for up to 12 hours post-infection (Rempel *et al.*, 1990). Thus it appears that [^{3}H]-thymidine incorporation measures only the initial stages of DNA replication. The reason for the failure of incorporation at later times during the infection is not clear, but it has been proposed that this failure is due to the depression of thymidine kinase activity in the infected cells by product inhibition (Traktman, 1990). Other potential explanations include dilution of the labelled nucleoside by expanded cellular pools and the inhibition of nucleoside uptake at late times during infection (Traktman, 1990). The cytoplasmic factories of viral DNA continue to expand throughout the course of the infection and continuous protein synthesis is required for the recruitment of nascent viral genomes from the nucleoprotein virosomes into immature virus particles (Esteban & Holowczak, 1978). It has been observed that inhibition of DNA synthesis at 6 hours post-infection appears to have no influence upon the final yield of infectious particles (Salzman *et al.*, 1963); thus it appears that most of the productive DNA synthesis occurs within the initial 5 hours of the infection.

(ii) DNA Synthesis In Vivo

Immediately following the rupture of the viral core (phase II uncoating) the viral genome is released into the cytoplasm and DNA synthesis is initiated. Evidence obtained by pulse labelling replicating viral DNA and analysis of the labelled restriction fragments suggests that DNA synthesis is initiated at or near the hairpin termini (Pogo *et al.*, 1981). The most heavily labelled restriction fragments are those obtained from either end of the genome, suggesting that replication can initiate at either terminus with an equal probability (Pogo *et al.*, 1981). This same study indicated that DNA synthesis proceeds along the genome and terminates at the opposite end rather than traversing around the hairpin termini. Further evidence for the initiation of DNA synthesis at the viral hairpin termini is derived from E.M. photographs of replicating viral DNA. Families of DNA molecules can be observed in infected cells with small double stranded loops at one end that appear to increase in size until the loop segregates into two progeny linear molecules (Esteban *et al.*, 1977). Viral DNA molecules apparently in the process of circularization have also been described by Holowczak (1982). Some of these E.M.s have been interpreted as showing the presence of replication forks in the circularizing molecule. In both of these E.M. studies the authors have indicated that the molecules described form a very small portion (2%) of the total replicating DNA. Thus interpretation of these DNA structures as replicative intermediates must be viewed with caution. The mechanism by which viral DNA synthesis is initiated may involve the formation of discrete, specific nicks at either or both termini. It has been shown that about 90 minutes after infection the viral DNA loses the covalent linkage at its termini as monitored by the loss of the hairpin terminal snap-back fragments (Pogo, 1977, 1980). While these observations may be an accurate assessment of the events that occur *in vivo* it is not clear that this represents a specific initiation event . It may be, for instance, that replication initiates at the first DNA sequence that becomes available, and the mechanism of core particle rupture most often leads to exposure of the termini before other parts of the genome. To date no viral primase function has been identified and so a nicking event of the kind described may function as an essential step in the formation of a free 3' hydroxyl to be used for priming DNA synthesis. At late times priming may occur by the numerous apparently random strand invasion events that occur among the replicating DNA molecules (Parks & Evans,1991a, 1991b, Fisher *et al.*, 1991).

The mode of poxvirus DNA replication has been extensively studied. However, many of these studies have been limited by the sensitivity of the techniques that were available at the time. As a result data pertaining to the the size of replicative intermediate DNA structures is in conflict with data obtained by using contemporary techniques. It is also not clear, for instance, whether the lagging strand DNA is initiated with an RNA primer, or if replication is bidirectional.

Density shifts of nascent 12 S DNA during isopycnic centrifugation in Cs_2SO_4 have been interpreted to suggest that some of the newly replicated viral DNA is covalently linked to RNA (Pogo & O'Shea, 1978). These data must be viewed critically due to the inherent difficulties in using buoyant density to identify a population of DNA molecules that have very short covalently linked RNA primers (Kornberg & Baker, 1991). Enzymatic digestion with spleen exonuclease has provided a second line of evidence that viral DNA synthesis is initiated with RNA primers. Most of the 12 S viral DNA becomes susceptible to the action of spleen exonuclease after alkaline hydrolysis (Pogo & O'Shea, 1978). This technique of identifying RNA tipped DNA strands is reliable with limited and clearly defined substrates (Sugino *et al.*, 1972, Kornberg & Baker, 1991). The accuracy of this technique is limited because 5'-hydroxyls (the target of spleen exonuclease) can be generated not only by alkaline hydrolysis of RNA primers but also by cleavage of apurinic/apyrimidinic sites and strand breakage at the site of misincorporated ribonucleotides (Sugino *et al.*, 1972, Kornberg & Baker, 1991). Thus, the conclusion that vaccinia DNA synthesis is RNA primed must await further investigation. It is clear, however, that the synthesis of viral DNA occurs in a discontinuous manner. This has been shown by pulse labelling replicating DNA and demonstrating that the label is initially incorporated into short DNA fragments that sediment at 8 to 20 S in alkaline sucrose gradients. Longer pulses have also shown incorporation into more rapidly sedimenting fragments, and perhaps most convincingly, the small pieces of DNA labelled following a short pulse could be chased into larger fragments (Holowczak & Diamond, 1976). Density labelling of replicating viral DNA with 5-bromo-2'-deoxyuridine (BrdUr) followed by isopycnic centrifugation indicates that the mode of replication is semiconservative (Esteban & Holowczak, 1977). Similarly, when cells are infected with virus that has label incorporated into its genomic DNA, the parental DNA is not degraded but is incorporated into the pool of replicating DNA.

Investigations into the nature of poxvirus replicative intermediates using alkaline sucrose gradient sedimentation indicated that no molecules greater than unit length were present in the infected cell cytoplasm (Holowczak & Diamond, 1976).A caveat to this interpretation is that DNA structures longer than genome size may be particularly susceptible to breakage. Indeed contemporary investigations have suggested that very long replicative intermediate structures do exist (see below and also section D iii). The inability to detect DNA molecules longer than a single genome on alkaline sucrose gradients lead to the probably erroneous interpretation that DNA synthesis is symmetrical and that no closed circular or concatemeric intermediates occur during the replication of viral DNA. It has been suggested that poxvirus

DNA replication proceeds in a bidirectional mode (Pogo & O'Shea, 1978). This interpretation was made based upon a decrease in size, following UV irradiation, of nascent poxvirus DNA that had been labelled sequentially with BrdUr and [^{3}H]-thymidine; a technique described by Weintraub (1972). To date no other attempts to distinguish between unidirectional and bidirectional replication in poxvirus DNA have been reported.

The rapidly sedimenting aggregates of replicating viral DNA first observed by Cairns (1960), were shown to be a network of nicked viral DNA molecules (Esteban & Holowczak, 1977). The replicating DNA complex is readily dissociated in the absence of Mg^{++} and at low ionic strengths indicating that the stability of the complex is dependent mainly upon DNA-DNA interactions (Dahl & Kates, 1970). Fractionation of the replicating forms of vaccinia virus DNA by neutral sucrose gradient sedimentation demonstrated that labelled nucleosides were first incorporated into a rapidly sedimenting (120 S) virosomal complex. This label could be chased from the rapidly sedimenting complexes into 90-96 S forms and then to the 68-72 S form corresponding to the mature viral DNA. Some intermediate forms sedimenting at 82-89 S were observed that were suggested as candidates consistent with the circularizing intermediate molecules (Holowczak & Diamond, 1976). A similar flow of viral DNA based upon sedimentation through neutral sucrose gradients was observed by Moyer & Graves (1981). They were able to identify the fractions as DNA in virosomal complexes that was first converted to DNA within immature viral particles (maximal conversion at 7.5 to 9 hours post-infection), and then mature virions (maximal recruitment from 14 to 24 hours post-infection). By using electrophoresis to analyze labelled restriction fragments of replicating rabbitpox virus DNA, these workers observed concatemers of viral genomes linked end-to-end. Both head-to-head and tail-to-tail linkages were observed along with the less frequent occurrence of head-to-tail linkages (Moyer & Graves, 1981). A similar finding was made for the replicative intermediate structures of vaccinia virus (Merchlinsky & Moss, 1989a). Unlike rabbitpox virus the vaccinia virus DNA was found to be composed of roughly equal proportions of head-to-head, tail-to-tail and head-to-tail structures (Merchlinsky & Moss, 1989a). That the concatemeric structure is a true replicative intermediate was substantiated by the identification and cloning of a DNA restriction fragment corresponding to the concatemer junction from vaccinia virus infected cells (Merchlinsky *et al.*, 1988). The observation of concatemeric replicative intermediates lead to a model for viral DNA replication that allowed the formation of nicked concatemeric molecules at least four genome lengths in size (Moyer & Graves, 1981). The mode of replication proposed by this model suggests that a replicative intermediate four genome lengths in size might be observable under nondenaturing conditions. The use of pulsed-field gel electrophoresis to fractionate the DNA intermediates in viral replication showed a rapid conversion of unit length genomes into high molecular weight structures many genome lengths in size (Merchlinsky & Moss, 1989a, DeLange, 1989). A portion of the high molecular weight DNA displayed mobility that was not consistent with linear molecules (Merchlinsky & Moss, 1989a, DeLange, 1989). These putative nonlinear structures may correspond to circular or branched DNA intermediate structures that may be important intermediates in the replicative pathway. No DNA structures of intermediate size can be distinguished electrophoretically during infections with wild type virus. However, a ladder of linear intermediate sized structures ranging from one to many genome lengths can be detected during infection under non-permissive conditions with vaccinia virus mutants that are conditional lethal for the expression of late genes (DeLange, 1989, Merchlinsky & Moss, 1989a).

Another model for poxvirus DNA replication that takes into account the viral hairpin termini and concatemeric intermediates is a self-priming model (Baroudy *et al.*, 1983, Moss, 1990a). Both of these models invoke site-specific nicking internal to the hairpin loop and unidirectional continuous or discontinuous DNA synthesis. In both cases strand displacement synthesis is invoked with the transient formation of single stranded DNA that can hybridize to other molecules.This type of a replication mechanism may account for the sedimentation behavior of the virosomal complexes and also the stimulation of homologous recombination with heteroduplex DNA formation within the infected cell cytoplasm (Ball, 1987, Evans *et al.*, 1988, Fisher *et al.*, 1991). The position of the initiating site-specific nicks of the self priming model also take into account the maintenance of the "flip" and "flop" isomers of the terminal sequences. During the process of maturation, concatemeric viral DNA is recruited from the virosomal pool into immature particles. The viral DNA found in the immature particles is the length of a single genome and lacks the restriction fragments that are diagnostic of head-to-head and tail-to-tail genome linkages (Moyer & Graves, 1981). Thus during the process of maturation the intermediate forms of viral DNA are cleaved and processed to yield unit length genomes. (The details of this process will be discussed in section D [iii]).

Specific origins of replication have been identified for many prokaryotic organisms, yeasts and the eukaryotic viruses SV40, papilloma viruses, adenovirus and some members of the herpesvirus family (reviewed by Kornberg & Baker, 1991). The observation that DNA synthesis appears to initiate at the termini of poxvirus DNA molecules suggests that the terminal sequences may act as origins of replication. Viral origins of replication are amenable to investigation by inserting the putative origin into a bacterial plasmid and then introducing the plasmid construct into virus infected cells. Virus specific replication of the plasmid can be scored by the appearance of *Dpn*I resistant plasmid sequences only in cells that are infected with virus. The restriction endonuclease *Dpn*I recognizes a sequence that is methylated in dam$^+$ E. coli strains but is not methylated following replication in mammalian cells. Thus replicated marker plasmid DNA can be distinguished from the input plasmid DNA by its resistance to cleavage by *Dpn*I. Experiments of this type have yielded the novel finding that closed circular DNA molecules are replicated in a sequence independent fashion in the cytoplasm of poxvirus-infected cells (DeLange & McFadden, 1986). The plasmid DNA is replicated into high molecular weight structures that restriction digests indicate are head-tail concatemers (DeLange & McFadden, 1986). The ability of plasmid DNA to be replicated in a sequence-nonspecific fashion in poxvirus infected cells suggests that no viral origin sequence is required for the initiation of poxvirus replication.These data do not exclude the presence of a specific viral origin sequence; however, they do make it difficult to identify the sequence without the use of 2-dimensional gel techniques to map initiation sites (Brewer & Fangman, 1987). Another finding from this type of experiment is that the replicated plasmid DNA is greater than unit length as demonstrated by electrophoresis through alkaline agarose gels. This result is at odds with the results found for viral DNA in alkaline sucrose gradients and suggests the possibility that in earlier studies much of the DNA was nicked by the preparation procedure or that the nascent DNA was not yet completely ligated at the time that it was collected. The other possibility is that the viral genomic DNA and the transfected plasmid DNA are replicated in a fundamentally different fashion.

(iii) DNA Synthesis In Vitro

The replication of adenovirus and SV40 DNA *in vitro* with extracts from infected cells or with purified proteins (Weinberg *et al.*, 1990) has yielded a wealth of knowledge about the mechanisms of eukaryotic DNA replication. These systems have been able to accurately mimic the initiation and elongation phases of the replication of small nuclear viruses. Attempts have been made to study the replication of vaccinia virus DNA *in vitro* using infected cell lysates (Esteban & Holowczak, 1977, Lambert & Magee, 1977) and permeabilized cells (Berger *et al.*, 1978). These studies have been able to demonstrate DNA synthesis dependent upon viral infection. The products synthesized are similar to the nascent DNA identified during *in vivo* replication, that is, label is initially incorporated into short fragments that elongate into DNA fragments that behave like full length viral DNA on sucrose gradients. No newly synthesized molecules longer than mature viral DNA could be identified suggesting that like the situation *in vivo* the DNA was synthesized discontinuously and in a symmetric fashion without any closed circular or concatemeric intermediates. Each of these systems were able to mimic DNA synthesis for about 30 minutes and complete the replication elongation that was in progress at the time of cell lysis; however, in no case were new rounds of synthesis initiated.

(iv) Homologous recombination in poxvirus infected cells.

Poxvirus DNA is subjected to extensive recombination during the replicative cycle. The high degree of viral recombination is evinced by the ability to demonstrate linkage of pock phenotypes in rabbitpox virus (Gemmel & Cairns, 1959) and by the ability to observe marker crosses during coinfections with two viruses carrying distinct genetic markers (Fenner & Comben 1958). Intermolecular and intramolecular recombination are stimulated in cells infected with vaccinia virus or SFV (Evans *et al.*, 1988 Merchlinsky, 1989). The very active poxvirus recombination system has proven useful for mapping viral genes by marker rescue (Nakano *et al.*, 1982, Weir *et al.*, 1982) and for the generation of recombinant poxvirus vectors (Mackett *et al.*, 1982). Poxvirus induced homologous recombination is dependent upon viral DNA synthesis (Ball, 1987, Evans *et al.*, 1988, Merchlinsky, 1989) but can proceed in the absence of late gene expression (Merchlinsky, 1989). The mechanism of viral recombination is clearly distinct from that of the host cell in that it appears to proceed through the generation of short stretches of heteroduplex DNA (Parks & Evans, 1991a, Fisher *et al.*, 1991). The formation of heteroduplex regions in poxvirus infected cells is marked by its high frequency of about one productive exchange per 500 bp (Parks & Evans, 1991a). Another feature of these virally induced exchanges of DNA that is unique from host cell recombination is the high negative interference displayed when closely spaced markers are crossed (Parks & Evans, 1991a). The poxvirus DNA polymerase is the only viral gene product thus far known to be involved in virus induced recombination. It may be that recombination is inextricably linked with DNA replication and so mutants will be difficult to identify because the phenotypes will be indistinguishable.

(v) Viral enzymes involved in DNA precursor biosynthesis.

The exclusively cytoplasmic location of viral DNA synthesis constrains poxviruses with a requirement to either encode and synthesize replicative enzymes or to recruit host enzymes from the nucleus. Initial evidence for virally encoded enzymes of DNA replication came from the observed induction of DNA polymerase (Magee, 1962, Green & Pina, 1962), thymidine kinase (Magee, 1962), DNA ligase (Sambrook & Shatkin, 1969, Spardi 1976) and deoxyribonuclease (Hanafusa, 1961) activities in infected cell lysates. These activities were subsequently shown to be virus specific based upon immunological distinction from host enzymes, isolation of genetic mutants, and finally by identification of the viral genes for many of the replicative enzymes (reviewed by Traktman 1990). Some of the enzymes known to be encoded by vaccinia virus and the relative location of the genes coding for them are illustrated in figure I-3. Also listed are some gene products that have been identified biochemically, but that have not yet been mapped on the viral genome.

Replication of DNA is dependent upon the availability of sufficient pools of precursor deoxyribonucleoside triphosphates. In eukaryotic cells the enzyme activities required for the synthesis of DNA precursors are expressed in late G1 and throughout S phase of the cell cycle (reviewed by Kornberg & Baker, 1991). Poxvirus DNA is replicated in an S-phase independent fashion in host cells and can replicate in enucleated cells (Hruby *et al.*, 1979). These two observations indicate that upon infection poxviruses can induce alterations in the cytoplasmic pools of DNA precursors by virtue of virally encoded enzymes. Deoxyribonucleoside triphosphate pools have been shown to enlarge upon infection with vaccinia virus (Slabaugh *et al.*, 1991). The increased precursor pool sizes occur concurrent with the induction of novel ribonucleotide reductase and thymidine kinase activities in the infected cell cytoplasms (Slabaugh *et al.*, 1984, Magee, 1962).

Ribonucleotide Reductase: The vaccinia virus ribonucleotide reductase functions as a heterodimer similar to other eukaryotic ribonucleotide reductases (Slabaugh *et al.*, 1988). Both subunits of the vaccinia virus ribonucleotide reductase are similar to the cellular versions; the small subunits share 80% homology at the amino acid level (Slabaugh *et al.*,

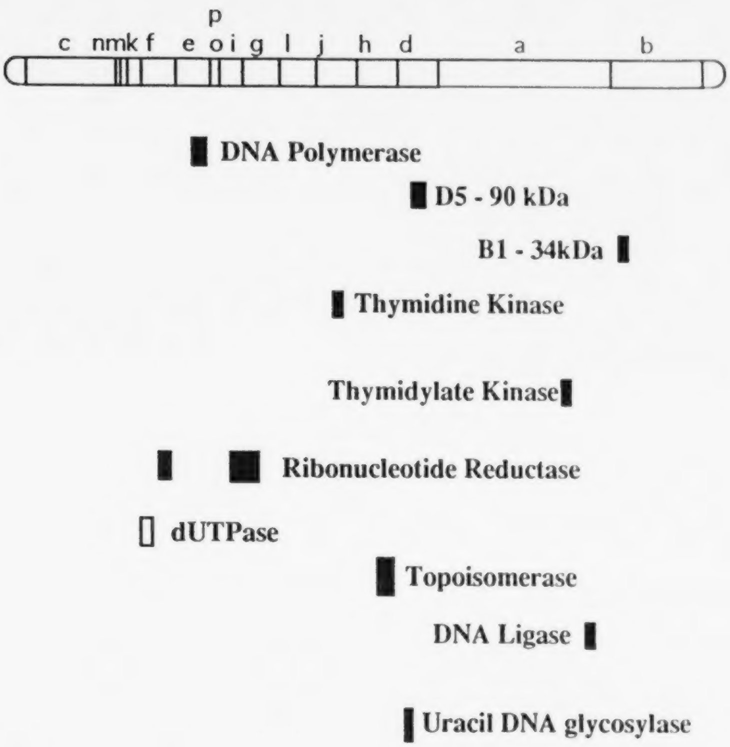
p
c nmk f e o i g l j h d a b
DNA Polymerase
D5 - 90 kDa
B1 - 34kDa
Thymidine Kinase
Thymidylate Kinase
Ribonucleotide Reductase
dUTPase
Topoisomerase
DNA Ligase
Uracil DNA glycosylase

Figure I-3. Enzymes involved in the replication of vaccinia virus DNA. The relative chromosomal position of the genes encoding vaccinia virus DNA repair enzymes are shown with blocks. The gene for dUTPase was identified by DNA sequence homology but no enzymatic activity for the dUTPase gene product has been demonstrated. The open reading frames D5, and B1 have been implicated in DNA replication by the inability of viruses carrying temperatures-sensitive alleles of these genes to synthesize DNA at the non-permissive temperature, the actual functions remain unknown. Topoisomerase has been demonstrated to be an essential gene, but an actual role in DNA synthesis is thus far only inferred. The uracil DNA glycosylase functions in excision repair; but is an essential gene because viruses that carry temperature sensitive alleles of the gene are unable to synthesize DNA at the non-permissive temperature. The genes that encode the nicking-joining enzyme and the other virally induced deoxyribonucleases have not yet been identified.

1988, Tenglesen *et al.*, 1988). Although the viral and cellular versions of ribonucleotide reductase are very similar they can be distinguished based upon differential sensitivity to Mg^{++}/AMP-PNP with regard to CDP reduction (Slabaugh & Mathews, 1984). The genes for both subunits of the viral enzyme are expressed early during infection, consistent with a role in the synthesis of precursors for DNA replication. Ribonucleotide reductase is strongly inhibited by hydroxyurea (HU) and this drug subsequently results in an inhibition of viral DNA replication due to a limitation of precursors. Overexpression of the small subunit of ribonucleotide reductase can confer resistance to HU. HU resistant mutant viruses have been identified in which the small subunit of ribonucleotide reductase has been duplicated to create tandem arrays of the small subunit gene (Slabaugh *et al.*, 1988). In the absence of selective pressure the tandem arrays are rapidly lost . Viral ribonucleotide reductase is non-essential for growth in tissue culture suggesting that the host enzyme can be recruited to synthesize precursors for viral DNA synthesis.

Thymidine kinase: Soon after infection of tissue culture cells with vaccinia virus (Magee, 1962) or SFV (Barbanti-Brodano *et al.*, 1968), a thymidine kinase (T. K.) activity is induced. The vaccinia virus T. K. gene has been mapped (Hruby & Ball, 1982) along with the T. K. genes of SFV (Upton & McFadden, 1986), variola and Monkey pox (Esposito & Knight, 1984). The 19 kDa gene product functions as a homotetramer that phosphorylates thymidine to provide precursors for DNA synthesis (Hruby, 1985). The T. K. gene is expressed exclusively during the early phase of the infection and the mRNA is no longer detectable 8 hours post-infection (Hruby & Ball, 1981). The enzyme is relatively stable and persists throughout the course of the infection, but the enzyme activity becomes depressed at late times in the infection. This may be related to the elevated cellular pools of dTTP (Slabaugh *et al.*, 1991) which are known to inhibit the viral T. K. activity (Hruby, 1985). Expression of the T. K. gene is non-essential for growth in tissue culture (Buller et al., 1985). Vaccinia virus mutants that are defective in the induction of T. K. activity have been selected by growth in T. K. deficient cell lines in the presence of BrdUr. This selection works because the viral T. K. will phosphorylate BrdUr thus allowing the lethal incorporation of the nucleotide analog into cellular and viral DNA. This particular observation has proven to be useful as a selection procedure in the generation of recombinant viruses carrying a foreign gene inserted into the viral T.K. locus (Mackett *et al.*, 1982). Poxviruses with null alleles of the T.K. gene replicate in tissue culture but are severely attenuated in animal hosts (Buller *et al.*, 1985). The reason for this difference is not entirely clear, but presumably it it related to the metabolic state of the infected cells with the primary tissue cells being mostly in a quiescent state while the cultured cells are continuously growing.

Thymidylate kinase: A gene in the vaccinia virus Hind III A fragment was identified by homology with the S. cerevisiae thymidylate kinase gene (Smith *et al.*, 1989). This enzyme could function in the pathway of dTTP synthesis immediately following the thymidine kinase activity. No induction of thymidylate kinase activity can be detected in vaccinia virus infected cells, but a recombinant form of the gene product has been shown to have thymidylate kinase activity *in vitro* (Hughes *et al.*, 1991). The gene is expressed at early times during infection and is not essential for growth in tissue culture (Smith *et al.*, 1989, Perkus *et al.*, 1991).

dUTPase: The F2L open reading frame of vaccinia virus encodes a gene with significant sequence homology to eukaryotic dUTPases. No activity has yet been demonstrated for the product of this gene. If the F2L does encode a dUTPase enzyme it would be expected to act in the synthesis of dTTP as has been shown for the dUTPase encoded by HSV (Roizman & Sears, 1990, Williams & Parris, 1987). Another function for dUTPase is to reduce the pool of dUTP which can be incorporated into DNA. The F2L open reading frame is not essential for growth in tissue culture (Perkus *et al.*, 1991).

Other activities potentially involved in precursor biosynthesis include pyrimidine nucleotide kinases and nucleoside phosphohydrolases. These activities may be encoded or host enzymes may be utilized.

(vi) Enzymes involved in DNA synthesis:

DNA polymerase: By virtue of the cytoplasmic site of DNA replication poxviruses are able to escape cellular S-phase regulation and are constrained to the use of a virally encoded DNA polymerase. Following infection with vaccinia virus a rapid induction of DNA polymerase activity can be detected in infected cells (Magee, 1962). The induced polymerase activity is immunologically distinct from host activities (Magee & Miller, 1967) and can be biochemically separated from host enzymes (Chalberg & Englund, 1979). The vaccinia virus DNA polymerase can be differentiated from the host cell activity by its sensitivity to phosphonoacetic acid (PAA), and the isolation of viral variants resistant to (PAA) suggested that the polymerase activity might be virally encoded (Moss & Cooper, 1982, Sridhar & Condit, 1983). The gene coding for the vaccinia virus DNA polymerase has been mapped on the genome to the Hind III E fragment (Jones & Moss, 1984, Traktman *et al.*, 1984). The polymerase is a 110 kDa protein that can function as a monomer with DNA polymerase activity and 3'-5' exonuclease (potentially "proof reading") activity. DNA polymerase activity is dependent upon the presence of a free 3' hydroxyl to function as a primer. The purified enzyme lacks processivity and is incapable of performing strand displacement synthesis or of proceeding through secondary structure in templates. This suggests that the process of DNA replication requires accessory proteins to allow the polymerase to traverse the genome. Vaccinia virus DNA polymerase shows significant homology to other B-type replicative DNA polymerases (Wong *et al.*, 1988, Ito & Braithwaite, 1991). The viral DNA polymerase gene is essential for virus growth and the enzyme activity has also been shown to be required for homologous recombination in infected cells (Ball, 1987, Evans *et al.*, 1988, Merchlinsky, 1989).

Consistent with its role in genome replication, the vaccinia virus DNA polymerase gene is expressed early during infection in a highly regulated fashion. The transcriptional regulatory signals appear to be complex including both early and late start signals and early termination signals within the open reading frame (reviewed by Traktman, 1990). A particularly unusual feature of the DNA polymerase gene is the transient nature of its expression. In the absence of DNA replication and late gene expression many early genes continue to be transcribed (Condit & Motyczka, 1981); however, the polymerase gene is transcribed only until about 3 hours post-infection at which time its synthesis decreases regardless of whether or not DNA replication and late gene expression is initiated (Traktman, 1990).

Vaccinia virus D5 ORF: Temperature-sensitive vaccinia virus mutants that are defective in DNA synthesis at the non-permissive temperature have been mapped to the Hind III D5 ORF (Evans & Traktman, 1987, Roseman & Hruby, 1987). The D5 ORF codes for a protein of approximately 90 kDa that is expressed at early times during the infection. Synthesis of the protein peaks at 2.5 hours post-infection and then declines even in the absence of DNA replication (Evans & Traktman, 1987). If D5 ts mutants are allowed to initiate DNA replication at the permissive temperature and then are shifted to the non-permissive temperature there is a very rapid cessation of the incorporation of $[^3H]$-thymidine into DNA suggesting that the D5 gene product functions at the replication fork and is essential for the elongation phase of DNA replication.

Vaccinia virus B1 ORF: Two vaccinia virus mutants that are deficient in DNA synthesis at elevated temperatures have been mapped to the B1 ORF. This gene codes for a 34 kDa protein that is expressed at early times in infection (Traktman *et al.*, 1989). The function of the 34 kDa protein is unknown but the amino acid sequence displays significant homology to a family of serine-threonine protein kinases (Traktman *et al.*, 1989). A peculiar feature of virus mutants in the B1 ORF is that the defect is in part dependent upon the cell line that is infected. In mouse L cells DNA synthesis by B1 ts mutants at the non-permissive temperature is less than 5% of that synthesized at the permissive temperature; however, in monkey BSC-40 cells at the nonpermissive temperature DNA synthesis can approach 65% of that which occurs at the permissive temperature (Rempel *et al.*, 1990). The cause of this behavior is unknown, but it has been suggested that a host protein from primate cells may be able to complement the defect (Rempel *et al.*, 1990).

Vaccinia virus D4 ORF: A temperature-sensitive mutation that confers a defect in DNA synthesis to the vaccinia virus IHD strain has recently been mapped to the D4 orf (A. M. DeLange, personal communication). The product of this orf has not been entirely characterized but at least one of its functions is that of a uracil DNA glycosylase. How the D4 gene product is involved in DNA synthesis has not been determined.

Topoisomerase: A type I topoisomerase activity is induced in enucleated cells following infection with vaccinia virus (Poddar & Bauer, 1986). The viral source of the induced topoisomerase was later verified by the presence of a type I topoisomerase encapsidated in vaccinia virus cores (Shaffer & Traktman, 1987). The gene for the vaccinia virus and SFV enzymes have been sequenced (Shuman & Moss, 1987, Upton *et al.*, 1989). No specific data have been published with regard to regulation of the poxvirus topoisomerase gene expression. It is presumed that the vaccinia and SFV genes are expressed at late times because both are preceded by signals for late transcription. Also consistent with expression at late times is the observation that the enzyme activities are induced following the initiation of DNA replication (Poddar & Bauer, 1986). The specific function of the viral enzyme is not known but it is believed that topoisomerase would function to relieve the torsional strain generated by transcription and/or DNA synthesis. The gene is essential for virus replication as deduced by the inability to generate insertional mutants (Shuman *et al.*, 1989).

DNA ligase: All known mechanisms of DNA replication and recombination require a DNA ligase activity to seal the strand discontinuities generated by either process. A DNA ligase activity that is capable of fulfilling this function in viral replication is induced by vaccinia virus infection (Sambrook & Shatkin, 1969, Spadari, 1976). A gene with homology to yeast DNA ligase was identified in the vaccinia virus WR Hind III A50 ORF (Smith *et al.*, 1989, Kerr *et al.*, 1989). The viral gene product was identified by demonstrating ligase activity from the RNA translated *in vitro*. The viral gene product has also been identified in infected cell lysates by virtue of its ability to form a covalent bond with $\alpha[^{32}P]$ labelled ATP (Colinas *et al.*, 1990). A peculiar observation is that the gene does not appear to be essential for the viral replication cycle in tissue culture. It has been postulated that vaccinia virus can utilize the host cell activity for this function (Colinas *et al.*, 1990) although the possibility that another virus gene can fulfil the ligase function cannot be discounted.

Viral DNases: Four deoxyribonuclease activities that are induced by vaccinia virus infection have been purified and characterized.The vaccinia virus DNA polymerase has a 3'-5' exonuclease activity that may function in "proof reading". A DNase activity with a pH optimum of 4.5 (acid nuclease) is induced following the initiation of DNA replication (Roseman-Hornbeak & Moss, 1974a, 1974b). The enzyme has been isolated from viral cores and functions primarily on ssDNA. The acid DNase may be the well characterized nicking-joining enzyme that can nick the single stranded regions of supercoiled plasmid substrates and will ligate a small percentage of the nicked molecules in an ATP independent fashion (Lakritz *et al.*, 1985, Reddy & Bauer, 1989). The activity of the purified 50 kDa enzyme can be potentiated by trypsinization which produces an active 44 kDa fragment (Reddy & Bauer, 1989) and also by heating virion preparations to 50°C (McAuslan & Kates, 1967).

Another distinct 50 kDa nuclease with specificity for single stranded DNA and a pH optimum of 7.8 (neutral DNase) is expressed at early times during the viral infection and may also be isolated from viral cores (McAuslan & Kates, 1967, Pogo & Dales, 1969, Pogo & O'Shea, 1977). A fourth DNase that acts exclusively on native duplex DNA with a pH optimum of 9.2 is expressed at early times in the infection. This nuclease has been identified only in infected cells and can not be detected in purified preparations of vaccinia virus cores (McAuslan & Kates, 1966). With the exception of the 3'-5' exonuclease of the viral DNA polymerase, no specific functions have been assigned to any of these nucleases and the genes that encode them have not yet been identified.

A number of other viral proteins have been identified that interact with viral DNA either in the virion or in the cytoplasmic virosome (reviewed by Traktman, 1990). These proteins appear to be largely structural in nature and may be required for maintaining the tertiary structure of viral DNA for packaging. In the virosome, some of these structural proteins may be involved in the formation of "scaffold-like" structures that might be required for accurate transcription and replication of viral DNA .

Some biochemical activities that are expected to be required for DNA replication have not yet been identified in infected cell lysates. An RNA primase activity is expected to be required to produce primers for DNA synthesis. No primer synthesizing enzymes have yet been identified; however, it has been suggested that the viral RNA polymerase may serve this function (Pogo & O'Shea, 1978). On the other hand viable models for poxvirus DNA replication that involve nicking and self priming or priming by strand invasion have been proposed and these models have no inherent requirement for a primase activity.

A helicase activity is presumed to be required in conjunction with the viral DNA polymerase in order to enhance the processivity of the replicative enzyme and to allow the polymerase to traverse regions of secondary structure such as the hairpin termini or the many small palindromes that make up the terminal region of SFV. To date no viral helicase has been identified, but the fast stop DNA synthesis mutants identified in collections of conditional lethal vaccinia virus mutants are potential candidates. It is also possible that the encapsidated viral topoisomerase might act as a helicase to unwind template DNA in front of the advancing DNA polymerase.

D. Telomeres.

(i) Telomere structure and function.

The terminal sequences of any linear chromosome must fulfill a special biological role in the replication and stability of the chromosome. Thus Muller (1938) used the term telomere to describe the chromosomal termini that have the special property of providing a functional "cap" to protect the free ends of chromosomes from deletion and rearrangement. The special name was given to the chromosomal termini after the observation that broken ends of chromosomes are able to fuse together in abnormal arrangements (Muller, 1938, McClintock, 1941). Free chromosomal ends are also prone to exonuclease attack and are highly recombinogenic (McClintock, 1941, Orr-Weaver *et al.*, 1981). Telomeres may also have sequence properties that allow them to function in establishing the tertiary arrangement of the interphase nucleus by mediating the interaction of chromosomes with the nuclear envelope (Agard & Sedat, 1983, Mathog *et al.*, 1984). In addition, telomere sequences have been implicated in mediating the association of homologous and non-homologous chromosomes in a "bouquet" arrangement at the nuclear envelope during the first meiotic division (Lipps *et al.*, 1982). Part of the protective property offered by telomeres may be due to associated structural proteins. Telomere binding proteins have been characterized from S. cerevisiae (Berman *et al.*, 1986) and Oxytrichia nova (Gottschling & Zakian, 1986). The function of these proteins is not known; however, they will protect the telomere sequences from nucleolytic degradation *in vitro*. The telomere binding protein RAP-1 from yeast binds to the telomere sequences with high affinity and also functions as a *trans*-acting transcriptional enhancer/silencer. The connection between the telomere function and transcriptional function is not clear but overexpression of RAP-1 leads to an elongation of the chromosomal telomeres while underexpression causes a decrease in telomere length (Chambers *et al.*, 1989, Conrad *et al.*, 1990). Cells carrying mutant forms of RAP-1 that are temperature-sensitive for transcriptional activation have short telomeres (Conrad *et al.*, 1990). RAP-1 mutants that are defective in the transcriptional silencing function have elongated telomeres (Sussel & Shore, 1991). The telomeres of numerous single cell eukaryotes are transcribed (Rudenko & Ploeg, 1989). However, no correlation has been made between transcription of the telomere sequences and the length or stability of the telomeres.

Telomere sequences may play their most profound role in DNA replication. A special mechanism is required in order to allow complete replication of the 5' ends of linear chromosomes. This is because all known replicative DNA polymerases require a preformed primer, in order to initiate DNA synthesis (reviewed by Kornberg & Baker, 1991). In most eukaryotic cells and nuclear DNA viruses the primers are supplied by short oligoribonucleotide chains that are degraded following their use (Tseng & Goulian, 1975, Tseng *et al.*, 1979, Reichard *et al.*, 1974). This creates a problem for the replication of the 5' end of a linear molecule since the degradation of the primer would leave the 3' end of the complementary strand exposed to nuclease attack and the sequence of the extreme 5' end would be uncopied and ultimately lost. As a result each end of the chromosome would become progressively shorter with each round of DNA replication (Watson, 1972).

Insights into the mechanism of telomere function both in the maintenance of chromosome stability and in the replication of chromosomal termini have been gained by investigating the conserved structure of these chromosomal elements. The initial detailed investigations of telomere sequences were directed at the linear extrachromosomal ribosomal DNA (rDNA) molecules of the ciliated protozoan Tetrahymena thermophila (Blackburn & Gall, 1978). The rDNA of Tetrahymena form individual chromosomes within the macronucleus, and each of these rDNA chromosomes have telomere sequences. The telomeres of the Tetrahymena rDNA were exploited because the high copy number (approximately 10^4/macronucleus) and small size (21 kbp) of the rDNA molecules allowed direct sequencing of the native telomeres (Blackburn & Gall, 1978). The telomeres of these nuclear DNAs carry a variable number (20-70) of tandem repeats of the sequence motif 5' $[C_4A_2/T_2G_4]$3'. Tandemly repeated sequences are a characteristic feature of eukaryotic telomeres, but the specific sequence of the repeated motif varies among organisms (see table I-1). Another conserved feature of telomeres is a conserved 3' single stranded overhang. The extreme terminal sequences of Oxytricha can be represented as follows;

$5'C_4A_4C_4A_4$.......

$3'G_4T_4G_4T_4G_4T_4G_4T_4$........

A common feature of telomere sequences illustrated by table I-1 is that one strand is typically C rich while its complementary strand is "G" rich. The "C" rich strand has numerous non-ligatable internal nicks and the distal sequences can be labelled by nick translation. The inability to ligate or end-label the terminal sequences suggests that they are inherently capable of forming an unusual structure (Blackburn & Gall, 1978, Emery &Weiner, 1981). The repetitive nature and "G+C" richness of the telomere sequences has lead to speculation about the formation of base tetrads within the 3' overhang (Williamson *et al.*, 1989, Sundquist & Klug, 1989, Zahler *et al.*, 1991). The behavior of telomere sequence oligonucleotides in non-denaturing gels suggests that the sequences fold into a more compact structure (possibly hairpins) that result in their greater than expected mobility (Henderson *et al.*, 1988). This physical evidence for hairpin structures is corroborated by NMR data that indicates the presence of strong intrastrand interactions among the "G" residues. It has been further speculated that strong interstrand interactions may be the cause of the end-to-end chromosome attachment that has been observed (Gottschling & Cech, 1984).

A special class of covalently closed hairpin telomeres have been identified in eukaryotic viruses such as parvovirus (Berns *et al.*, 1985), iridoviruses (Gonzales *et al.*, 1986) and poxviruses (Geshelin & Berns, 1974, Baroudy *et al.*, 1982, DeLange *et al.*, 1986). Covalently closed hairpin termini have also been found on the linear plasmids of S. cerevisiae (Kikuchi *et al.*, 1985) and Rhizoctonia solani (Miyashita *et al.*, 1990), Paramecium mitochondrial DNA (Pritchard & Cummings, 1985), linear plastids of barley (Ellis & Day, 1986), and prokaryotic plasmids (Barbour & Garon, 1987). It appears that the structure of the hairpin telomere rather than a reiterated motif endows the terminal sequence with its ability to stabilize the chromosome and allow complete replication of the termini.

Oraganism	Sequence Repeat
Protazoans	
Tetrahymena	5' C_4A_2 3'
Paramecium	5' $C_3\text{C/A}A_2$ 3'
Glaucoma	5' C_4A_2 3'
Oxytricha	5' C_4A_4 3'
Euplotes	5' C_4A_4 3'
Stylonchia	5' C_4A_4 3'
Sporozoans	
Plasmodium	5' $C_3T\text{A/G}AA$ 3'
Flagellates	
Trypanosoma	5' C_3TA_2 3'
Yeasts	
Saccharomyces	5' $C_{2\text{-}3}A(CA)_{1\text{-}3}$ 3'
Schizosaccharomyces	5' $C_{1\text{-}6}G_{0\text{-}1}T_{0\text{-}1}GTA_{1\text{-}2}$ 3'
Slime molds	
Dictyostelium	5' $C_{1\text{-}8}T$ 3'
Didymium	5' C_3TA_2 3'
Plants	
Arabidopsis	5' C_3TA_3 3'
Mammals	
Human	5' C_3TA_2 3'

Table I-1 A compilation of the known repeat sequences that form the telomeres of a variety of eukaryotic organisms (adapted from Zakian, 1989).

(ii) Telomere replication

The requirement for a pre-existing 3' hydroxyl to prime DNA synthesis constrains the options for viable strategies that will allow chromosome replication without a loss of genetic information. Prokaryotic organisms and many eukaryotic viruses have adopted circular genomes that can be replicated without the need for a special mechanism to synthesize termini. The linear DNA genome of bacteriophage lambda undergoes a transient circularization to allow continuous replication around the genome creating multiple genome length molecules. The linear genomes of bacteriophages T4 and T7 undergo concatemerization during infection. This allows the ends of the chromosome to be replicated by DNA synthesis primed internally from an adjoining genome (Broker, 1973). The bacteriophage Ø29 and the mammalian adenoviruses overcome the problem of 5' end replication by the use of a protein primer that binds to the terminal genomic sequences and allows initiation of DNA synthesis (Harding & Ito, 1980, Rekosh *et al.*, 1977). Another solution to the problem of replicating chromosome ends is the use of a preformed RNA primer. Retroviruses prime the reverse transcription of their RNA genome with a cellular tRNA molecule (Verma *et al.*, 1971).

Covalently closed hairpin telomeres allow replication to proceed uninterrupted through the end of the chromosome with no need for a special priming mechanism. The resulting replicative intermediate does, however, require a special mechanism to resolve the telomere "fusion" into daughter hairpin terminated molecules.

Numerous mechanisms have been proposed to account for telomere replication (see Fig. I-4). Based on bacteriophage models, Watson (1972) proposed that all linear chromosomes must have terminal redundancies in order to facilitate circularization or concatemerization. Another model proposed that telomere sequences were palindromic (two fold symmetric) so that the unreplicated 3' sequences could fold back into a transient hairpin structure to prime DNA synthesis (Cavelier-Smith, 1974). This model requires a site or sequence-specific nuclease to nick the opposite strand to allow chromosome segregation and the

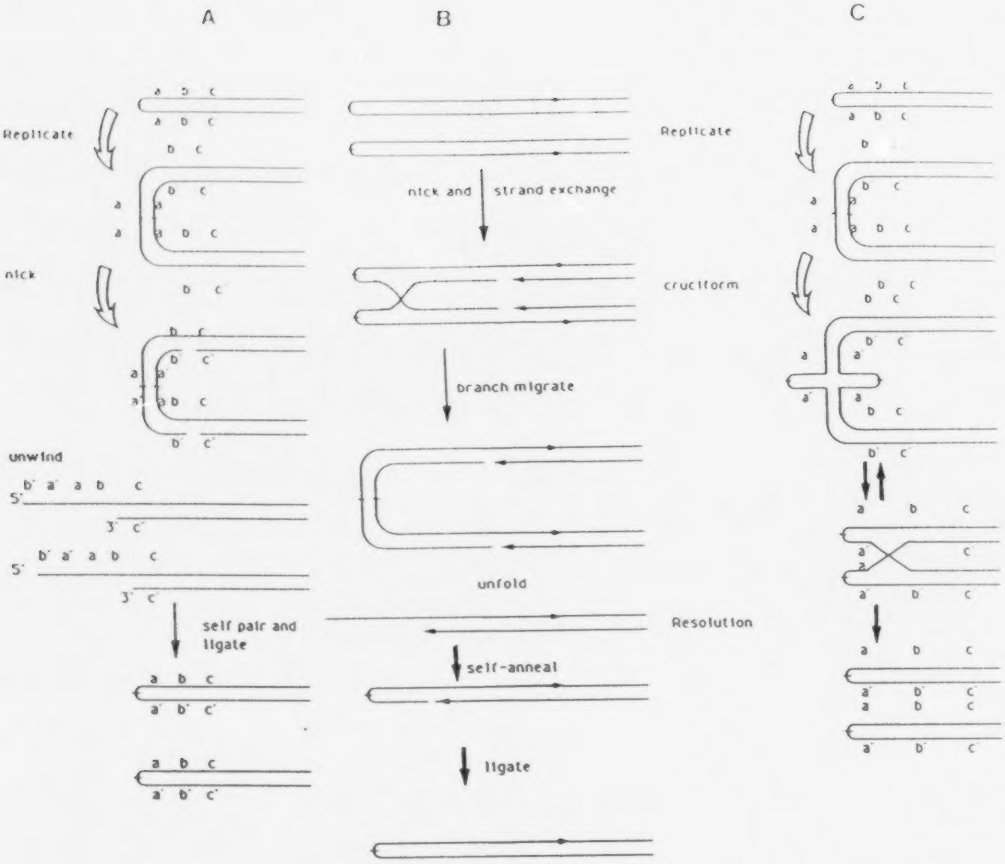
A
B
C
Replicate
nick
unwind
5'
3'
self pair and ligate
a b c
a' b' c'
nick and strand exchange
cruciform
branch migrate
unfold
self-anneal
ligate
Replicate
cruciform
Resolution

Figure I-4. Models of hairpin telomere replication and resolution. (A). The model of Bateman. (B). The Dancis and Holmquist model. (C). The cruciform extrusion model. Models A and C call for replication through the terminal hairpin structure with the concomitant generation of an inverted repeat intermediate structure. The replicative intermediate is then resolved into progeny hairpin termini by site-specific nicking followed by unwinding and refolding (Bateman, 1975). In the cruciform extrusion model, resolution occurs by the cleavage of an extruded cruciform by a Holliday junction endonuclease. Model B invokes recombination between telomere structures of different chromosomes to allow complete replication of the genomic termini. Resolution of the replicative intermediate is through a mechanism similar to that proposed by Bateman. Complementary sequences are indicated by a/a'. (adapted from McFadden & Dales, 1982).

complete replication of the telomere sequences. In a simplification of this model Bateman (1975) proposed that the palindromic telomere sequence could be a covalently closed hairpin. Replication through the hairpin would create a "telomere fusion" (actually a large palindrome), that requires resolution and the separation of daughter molecules. The original model called for: resolution via strand-specific nicks, separation of the daughter molecules, and hairpin formation by intrastrand folding to form new hairpin molecules. The Bateman model is relevant to the replication of the hairpin termini that exist in poxviruses and linear yeast plasmids. The Cavelier-Smith model more accurately accounts for the replication of eukaryotic parvoviruses that possess terminal palindromic sequences that fold into hairpin structures (Berns *et al.*, 1985). The validity of the model in the case of parvoviruses has been borne out by the demonstration of *in vitro* resolution of the replicative intermediates (Snyder *et al.*, 1990). Another model for the maintenance of telomere sequences invokes homologous recombination to create transient inverted repeats that can be resolved similar to the Bateman model (Dancis & Holmquist, 1979). Evidence for recombination between the telomere sequences of homologous and non-homologous chromosomes has been observed in yeast (Pluta & Zakian, 1989). Indeed it has been found that yeast possess a special mechanism for the resolution of telomere fusions (Szostak, 1983, Lundblad & Szostak, 1989).

A variation of the Bateman model has recognized the potential for palindromic intermediates created by replication through a hairpin or by homologous recombination between telomere sequences to freely branch migrate under the influence of superhelical tension (McFadden & Morgan, 1982, Szostak, 1983). In this model, the replicative intermediate can transiently extrude into a cruciform with the symmetry axis forming the apex of two hairpin structures. The extruded cruciform DNA can then be resolved by a Holliday junction endonuclease that would cleave the structure by nicking opposing strands at the base of the four-way junction.

An unexpected mechanism for the formation and maintenance of telomere sequences was first suggested by the ability of a linear molecule bearing Tetrahymena termini to replicate in yeast and during the process to acquire yeast telomere sequences (Shampay *et al.*, 1984, Szostak, 1982, 1983). This initial observation lead to the identification of a template independent telomere terminal transferase in cell free extracts from Tetrahymena (Greider & Blackburn, 1985). The enzyme activity can add telomere sequence repeats to the ends of chromosomes in a template independent fashion but requires a primer that is composed of at least two tandem repeats of telomere sequence. Since the initial observation the "telomerase" activity has been identified in Oxytricha (Zahler & Prescott, 1988), Euplotes (Shippen-Lentz & Blackburn, 1989), humans (Morin, 1989), and possibly yeast where mutants that fail to maintain telomere length have been identified (Lundblad & Szostak, 1989). The sequence specific addition of telomere repeats has been found to be template directed by an RNA molecule that forms an intrinsic part of the telomerase enzyme. The protein components of the telomerase have not yet been characterized in detail due to their apparent instability (reviewed by Blackburn, 1990). The temporal fluctuation of telomerase activity appears to be responsible for the variable length of the telomeres at various stages of the growth cycle of some organisms (Bernards *et al.*, 1983, Larson *et al.*, 1987, Forney & Blackburn, 1988). Detailed analysis of the telomerase activity has lead to a model for telomere replication in which the telomere sequences are dynamic in nature. During each round of DNA replication some telomere repeats are lost and these sequences are replaced by the telomerase activity. Shortening of telomere sequences has been associated in senescence in yeast (Lundblad & Szostak, 1989), Paramecium (Yu *et al.*, 1990) and human fibroblasts (Harley *et al.*, 1990).

(iii) Replication of Poxvirus telomeres

The autonomous nature and cytoplasmic location of DNA replication makes poxviruses a useful system for studying the replication and resolution of hairpin telomeres. Concatemeric intermediates consisting of multiple genomes linked through telomere fusions are *bona fide* replicative intermediates in poxvirus DNA replication. The telomeres of vaccinia virus (DeLange *et al.*, 1984, Merchlinsky & Moss, 1986) and SFV (DeLange *et al.*, 1986) were cloned by the method described by Szostak & Blackburn (1982). The cloned SFV telomere sequences were stable when propagated as inverted repeats in S. cerevisiae and in an E. coli strain (rec A, rec B, rec C, sbcB, sbcC) (DeLange *et al.*, 1986); however, deletion products are consistently generated (Dickie *et al.*, 1987). The vaccinia virus telomere sequence could be stably propagated in a recA E. coli strain (Merchlinsky & Moss, 1986). The ability to propagate cloned poxvirus telomere sequences in E. coli suggests that the inverted repeat was not extruded as a cruciform *in vivo*. The difference in stability between the vaccinia virus and SFV telomere sequences in E. coli may be due to the greater number of extra-helical bases present within the hairpin termini of vaccinia virus (DeLange *et al.*, 1986). It has been predicted that the extra-helical bases would increase the energy required for extrusion of the inverted repeat into a cruciform (Dickie *et al.*, 1987). Bacterial plasmids that contain a cloned inverted repeat version of the viral telomere are replicated into high molecular weight concatemers when transfected into poxvirus infected cells (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). These concatemeric structures are subsequently resolved into monomer length linear plasmids with viral hairpin termini (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). Concurrent DNA replication is not required for the resolution of the replicative intermediates; however, resolution displays a stringent requirement for late gene products i.e. those synthesized after the onset of DNA replication (Merchlinsky & Moss, 1989a). Resolution of the poxvirus replicative intermediate is a sequence dependent event. Unidirectional deletion analysis of the SFV and vaccinia virus replicative intermediates indicated that a 20 base pair sequence shared between SFV and vaccinia virus is required in *cis* to catalyze resolution of the replicative intermediate (DeLange & McFadden, 1986). The same sequence was subsequently shown to be conserved in other poxviruses (Merchlinsky, 1990). Conservation of the essential sequence and the ability of cloned vaccinia virus telomere replicative intermediates to be replicated and resolved in SFV infected cells indicates that there is a conserved mechanism for telomere resolution among poxviruses. (DeLange *et al.*, 1986). Detailed analysis of the vaccinia virus resolution sequence by site-specific mutagenesis revealed that the resolution sequence functions as a tripartite signal with conserved 5' TTTTTTT 3' and 5' TAAAT 3' sequence motifs separated by a non-conserved 9 to 11 basepair spacer (Merchlinsky & Moss, 1989b, Merchlinsky, 1990).

A genetic attempt to identify viral gene products involved in the resolution of vaccinia virus replicative intermediates has identified an number of conditional lethal viral mutants (Merchlinsky, 1989, DeLange, 1989). All of the mutants that are competent for DNA replication, but defective in telomere resolution, display pleiotropic defects related to late gene expression (Merchlinsky, 1989, DeLange, 1989). Most of these are mutants in RNA polymerase subunits and one, ts 22, synthesizes but rapidly degrades mRNA from late genes (Condit &Motyczkza, 1981, Pacha & Condit, 1985). One mutant that appears to be competent for late gene expression but defective in telomere resolution is ts 9383. This mutant has a single nucleotide substitution that leads to a single amino acid change in the small subunit of the mRNA capping enzyme (Carpenter & DeLange, 1991).

Resolution of the viral inverted repeat *in vivo* maintains the "flip" and "flop" configuration of the telomere extra helical bases. This result can be explained by either a Bateman model or a model that invokes the transient formation of a cruciform replicative intermediate. Another model that has been put forth to explain poxvirus telomere resolution invokes site-specific recombination between the inverted resolution sequences within the replicative intermediate (Merchlinsky, 1990).

The biochemistry of telomere resolution is poorly understood. The only gene product that has shown potential for involvement in the process is the viral nicking-joining enzyme; however, the specificity of the cleavages generated by this enzyme are not consistent with the products of *in vivo* resolution.

The Thesis Proposition.

The material presented in this thesis at first glance appears to be of a rather diverse and disconnected nature. A deeper consideration of the subject matter reveals that the single thread of DNA replication winds through the thesis linking together topics of replication, homologous recombination, DNA topology, transcription, and the acquisition of DNA precursors in the infected cell. It is necessary to consider all of these aspects of nucleic acid metabolism in an integrated fashion in order to address the major question of the mechanism of viral replicative intermediate resolution. Considering the problem out of context becomes difficult because the virus appears to utilize individual enzymes in numerous different roles.

Two sets of hypotheses have been considered: the first series deals directly with the question of poxviral replicative intermediate resolution and the second set addresses some other aspects of nucleic acid metabolism in poxvirus infected cells.

(a):

(1) The structure, and particularly the palindromic nature, of the viral replicative intermediate is a significant consideration that influences the mechanism of telomere resolution in a fashion that is independent of the sequence specific initiation of the resolution event.

(2) The sequences required in *cis* to catalyze the resolution of poxviral replicative intermediates act as viral late promoters.

(3) The transcriptional apparatus is directly involved in the resolution of viral replicative intermediates, but its role in this process is distinct from its ability to actively transcribe the terminal sequences.

(b):

(4) Poxvirus infection induces homologous recombination that is dependent upon viral DNA synthesis.

(5) Poxviruses encode a specific DNA repair mechanism in the form of a uracil DNA glycosylase.

Investigations into the mechanism of poxvirus replicative intermediate resolution that are presented in this thesis are largely dependent upon the ability of cloned versions of the poxvirus replicative intermediates to be replicated and resolved when transfected into poxvirus infected cells (DeLange *et al.*, 1986). Thus an inherent limitation and assumption of most of the work presented here is that plasmid DNA that is introduced into the poxvirus infected cells is replicated and processed in a fashion identical to the infecting viral DNA not only in regard to mechanism but also within the same temporal boundaries. A corollary to this is the assumption that the transfection procedures employed in these studies do not substantially influence the process of the viral infection within the transfected cells.

Cloned versions of poxvirus replicative intermediates were used throughout this study in an *in vivo* transfection assay that was developed previously (DeLange & McFadden, 1986). The availability of the cloned substrate molecule and the transient assay for telomere resolution allowed investigations using modified substrate molecules that could be used to identify significant features of the spacing, structure, and sequence of the poxvirus replicative intermediate and the effects of these parameters on resolution of the replicative intermediate *in vivo*. A series of insertion mutants developed and tested in chapter II provided information about the resolution process and thus set limits on the number of valid models that could be applied to poxvirus replicative intermediate resolution.

A report that poxvirus telomeres were transcribed at late times during infection (Parsons & Pickup, 1990) stimulated an investigation of the involvement of transcription and the transcriptional apparatus in viral telomere resolution. These studies, reported in chapter III, were aided not only by the *in vivo* resolution assay but also by the general utility of the poxvirus system whereby transcription can be easily assayed in a transient expression system *in vivo* and by the ability to manipulate the poxvirus genome by incorporation of sequences of interest into recombinant viruses. The rather unexpected finding that the poxviral DNA sequences that are required for replicative intermediate resolution also act as strong viral late promoters lead to further investigations, presented in chapter IV. In these studies an attempt was made to uncouple the processes of transcription and telomere resolution with metabolic inhibitors and site-specific mutations within the telomere resolution sequences.

A major limitation to all investigations that utilized the *in vivo* transfection assay was the inability to separate viral processes of DNA replication, recombination, and transcription. While this situation is really just a reflection of the complex series of interactions by which all aspects of viral nucleic acid metabolism are intertwined it seriously complicated the interpretation of results. In an attempt to overcome these problems and examine the process of telomere resolution in relative isolation an ongoing attempt to develop an *in vitro* system for telomere resolution is reported in chapter V. The progress made up to this point is largely due to the previously described implication of transcription in telomere resolution. Most success to date has been derived from cytoplasmic or whole cell extracts that are competent for the selective transcription of viral late genes. While the *in vitro* system has thus far provided only limited insight into the mechanism of telomere resolution it provides a starting place for the development of a more efficient extract and the isolation of individual activities by conventional biochemical complementation and purification techniques. Hopefully the use of wild type and mutant forms of the cloned replicative intermediate in this system will reveal more details about the resolution mechanism.

A series of other studies were undertaken that do not apply specifically to the problem of telomere resolution but are related by way of their involvement in viral nucleic acid metabolism. Homologous recombination has a major influence on the configuration of the viral DNA and upon the genetic diversity of the poxviruses as well as being a useful feature that allows the mapping of viral genes and the generation of recombinant viruses for expression or vaccine vectors. Initially we thought that viral enzymes that catalyze homologous recombination might be involved in telomere resolution. It is now clear that the processes of homologous recombination and telomere resolution are distinct; however, some of the activities involved in recombination may also play a role in resolution, chapter VI describes the development a plasmid model that was used to study recombination in poxvirus infected cells.

A completely different line of investigation lead to the discovery and characterization of a poxvirus encoded DNA repair enzyme that has an essential role in the replication of viral DNA (chapter VII). The identification of a viral DNA repair enzyme was not entirely unexpected since an enzyme of this type has been reported in herpes viruses; however, the essential nature of the gene encoding this ubiquitous function is thus far a novel finding.

Finally chapter VIII summarizes the conclusions based upon experimentally derived evidence that pertains to the resolution of poxvirus replicative intermediates, and the relevance of all of the models of telomere resolution that are consistent with the available experimental data are discussed.

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Chapter II

In vitro Mutagenesis of Cloned Poxvirus Replicative Intermediates Identifies Structural Constraints to Telomere Resolution

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A. Introduction

The requirement of all known replicative DNA polymerases for a free 3'-hydroxyl to prime the synthesis of a DNA strand necessitates a special mechanism for the faithful and complete replication of the 5' ends of linear DNA chromosomes (Watson, 1972). Many prokaryotes and some eukaryotic viruses have overcome this potential limitation by adopting closed circular genomes. Hairpin termini represent another strategy for circumventing the problem of replicating genomic termini (Bateman, 1975). Covalently closed, hairpin telomere structures have been observed in Tetrahymena rDNA (Blackburn & Gall, 1978), Paramecium mtDNA (Pritchard & Cummings, 1982), linear plasmids of yeast (Kikuchi *et al.*, 1985), plastids of barley (Ellis & Day ,1986) and the viral DNA genomes of parvoviruses (Berns *et al.*, 1985), iridoviruses (Gonzales *et al.*, 1986), and poxviruses (Geshelin & Berns, 1974, Baroudy *et al.*, 1982, DeLange *et al.*, 1986). Poxvirus DNA provides an appealing model system in which to study the replication and resolution of hairpin terminated DNA molecules. The poxvirus replicative cycle proceeds exclusively within the host cell in cytoplasmic factories or "virosomes" (Dales & Pogo, 1981, Holowczak, 1982) with little or no nuclear involvement. In fact most of the poxvirus replicative cycle including DNA synthesis can take place in enucleated cells (Hruby *et al.*, 1979, Prescott *et al.*, 1971). Thus it is believed that all of the proteins required for replication of poxvirus DNA are virally encoded. Another advantage of the poxvirus system is that the viral DNA is subjected to extensive recombination under the regulation of viral gene products (Fenner & Comben, 1959, Ball, 1987, Evans *et al.*, 1988). Thus it is possible to isolate mutants and to map the defective gene products via genetic marker rescue (Nakano *et al.*, 1982).

Shope fibroma virus, a virus of the genus Leporipoxvirus has a 160 kbp linear, double-stranded DNA genome with terminal inverted repeats (TIRs) of 12.4 kbp and covalently closed hairpin termini (DeLange *et al.*, 1984b, DeLange *et al.*, 1986). An unusual feature of the termini of SFV and other poxviruses is the presence of conserved extrahelical bases, 12 in the vaccinia virus WR strain (Baroudy *et al.*, 1982, Pickup *et al.*, 1983) and 8 at the terminus of SFV (DeLange *et al.*, 1986). These characteristic extrahelical bases within the TIRs of SFV and vaccinia virus result in two forms of the hairpin termini designated "flip" and "flop", that are distinguishable by sequence and differing mobility during native gel electrophoresis (Baroudy *et al.*, 1982). Replication of poxvirus DNA results in the generation of high molecular weight concatemers of viral DNA (Moyer & Graves, 1981, DeLange 1989, Merchlinsky & Moss, 1989a). Restriction enzyme analysis of the intramolecular forms of vaccinia virus DNA have demonstrated that the concatemers are linked end-to-end via a fusion of the terminal hairpin sequences (Moyer & Graves, 1981, 1986, Merchlinsky *et al.*, 1988). The sequence that forms the telomere fusion of SFV and vaccinia viruses has been cloned (DeLange *et al.*, 1984a, 1986) by a modification of the telomeric fusion protocol developed for Tetrahymena (Szostak & Blackburn, 1982). The same telomere fusion sequence has been cloned as a restriction fragment directly from DNA isolated from vaccinia virus infected cells (Merchlinsky *et al.*, 1988). DNA sequence analysis revealed that the palindromic fusion fragment was a precise duplex copy of the mature hairpin terminus; with each strand corresponding to one of the alternative "flip"-"flop" sequence conformations (DeLange *et al.*, 1986, Merchlinsky *et al.*, 1988).

Cloned versions of the inverted repeat telomere replicative intermediate are replicated into high molecular weight concatemers when transfected into poxvirus infected cells (DeLange et., 1986, Merchlinsky & Moss, 1986). These concatemeric molecules are subsequently resolved into monomer size linear minichromosomes with viral hairpin termini (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). The *in vivo* replication and resolution of circular plasmids bearing the viral telomere sequence lends credence to the hypothesis that the inverted repeat version of the viral telomere structure is a *bona fide* replicative intermediate. Covalently closed supercoiled or relaxed circular plasmids can be replicated into high molecular weight concatemers in a sequence non-specific fashion when transfected into

SFV infected cells (DeLange & McFadden, 1986). This finding was in conflict with results obtained by transfecting plasmid DNA into vaccinia virus infected cells. It was initially reported that plasmid DNAs lacking poxviral DNA sequences were not replicated in vaccinia virus infected cells. It was also reported that supercoiled plasmids containing poxvirus DNA were replicated ten times more efficiently than identical plasmids that had been relaxed with topoisomerase I (Merchlinsky & Moss, 1986). It was later demonstrated that replication of plasmid DNA transfected into poxvirus infected cells is sequence non-specific and is not enhanced by the presence of any poxvirus DNA sequences (DeLange *et al.*, 1986, Merchlinsky *et al.*, 1988). Resolution of the replicated concatemeric DNA was found to be dependent on the presence of specific poxviral sequences near the axis of symmetry in the palindromic replicative intermediate (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). The cloned replicative intermediates of SFV DNA can be replicated and resolved by the viral *trans*-acting factors present in both Leporipoxvirus and Orthopoxvirus infected cells (DeLange *et al.*, 1986). This observation has provided a system that can be manipulated at the molecular level to define the viral *cis*-acting sequences involved in the resolution of high molecular weight replicative intermediates. Analysis of the DNA sequences near the termini of SFV and vaccinia virus reveal a single region of highly conserved nucleotide sequence (DeLange *et al.*, 1986). Deletion analysis of cloned versions of the telomere replicative intermediates of both SFV and vaccinia virus has demonstrated that the sequences conserved between SFV and vaccinia virus are essential for the resolution of the inverted repeat into linear hairpin terminated minichromosomes (DeLange *et al.*, 1986). Extensive deletion analysis and point mutagenesis has defined the core sequence required for resolution of replicative intermediates to be the 20 nucleotide sequence ATTTAN$_{7-9}$AAAAAAA which occurs near the hairpin terminus (DeLange & McFadden, 1987, Merchlinsky & Moss, 1989b). The 20 bp core sequence is referred to as the telomere resolution target (TRT) (DeLange & McFadden, 1990). The TRT is conserved in sequence and in genomic arrangement among all of the poxviruses that have thus far been studied (Merchlinsky & Moss, 1989b, Merchlinsky, 1990). In the case of SFV some evidence indicates that sequences 5' to the TRT may have an enhancing effect upon the resolution of the replicative intermediate structures (DeLange *et al.*, 1986), but deletion analysis indicates that they are not essential and their role in resolution remains to be investigated.

The inverted repeat structure of the poxvirus telomere replicative intermediate places two copies of the core TRT sequence in an inverted orientation about one hundred nucleotide pairs apart. In an effort to define the structural requirements for telomere resolution, a cloned version of the SFV replicative intermediate has been subjected to extensive directed *in vitro* mutagenesis. A series of heterologous DNA sequence insertions and inversions of the native sequence have defined a requirement for the replicative intermediate to exist as a perfect or near perfect inverted repeat with two copies of the TRT sequence inverted with respect to one another and in a specific orientation with regard to the axis of dyad symmetry. These findings have allowed the formulation of several models for the resolution of poxvirus replicative intermediates.

B. Materials and Methods

Cells and Viruses. BGMK (African green monkey) and SIRC cells (rabbit corneal fibroblasts) were obtained from the American Type Culture Collection (ATCC) and were propagated as monolayer cultures in Dulbeco modified Eagle's medium (DME; Gibco laboratories) supplemented with 10% newborn calf serum (Gibco Laboratories).

Shope fibroma virus (SFV) and Myxoma virus were obtained from the ATCC and were propagated by low multiplicity infection of BGMK monolayers. Virus stocks were prepared from cell monolayers that had been infected for 48 hrs. The infected cell monolayers were scraped from the flasks and pelleted by centrifugation. Virus was released from the cell pellet by swelling followed by Dounce homogenization. The virus was separated from nuclei and cellular membranes by low speed centrifugation (Wills *et al.*, 1983). Virus titres were determined by infecting monolayers of 1.6 x 10^6 BGMK cells with 10 fold serial dilutions of crude virus stock in a 200 µl volume. Following a 1 hr adsorption at 37°C fresh medium was added to the monolayers. The infection was allowed to proceed for 2 days (vaccinia virus), or 4 days (SFV and myxoma virus), at which time the cells were fixed and stained with 0.1% crystal violet. Plaques (vaccinia virus) or foci (SFV and myxoma virus) were then counted to determine the virus titres.

Strains and Plasmids. All bacterial plasmids that contained inverted repeats of viral DNA sequence were maintained in Escherichia coli (E. coli) DB1256 (DeLange *et al.*, 1986). All other plasmids were propagated in E. coli strain HB101. Plasmids that contain large inverted repeats of SFV telomere sequence (pSCB1a, and pSAIB-56a) were derived by removing the appropriate *Cla*I or *Acc*I fragment from the plasmid pYSF1-30. Each fragment was subcloned into the *Sma*I site of pUC-19, as described previously (DeLange *et al.*, 1986). pSD-19 was derived as a unidirectional deletion of one side of the inverted repeat insert of pSAIB-56a and pSAD-2 was derived by deletion of 86 bp of sequence from the symmetry axis of the inverted repeat insert in pSD-19 (DeLange *et al.*, 1986). Linker insertion mutants of pSAD-2 were generated by relaxing the supercoiled DNA overnight with calf thymus topoisomerase I (a gift from Dr. A. R. Morgan) and cleaving the relaxed DNA at the unique *Afl*II restriction site present at the axis of dyad symmetry within the insert. The digested linear plasmid was purified away from the circular molecules by preparative gel electrophoresis and then blunt ends were created by treating the DNA with T4 DNA polymerase in the presence of 250 µM each of dATP, dTTP, dCTP, dGTP. The blunt ended vector was then ligated with T4 DNA ligase in the presence of a molar excess of 10 bp double stranded oligonucleotide *Bam*HI linkers that had phosphorylated 5' termini (Alberta regional DNA synthesis facility). Mutant clones were obtained by transforming competent DB1256 and screening the plasmid DNA obtained from ampicillin resistant colonies by cleavage with *Eco*RI/*Bam*HI and fractionating the digestion products on an 8%

non-denaturing polyacrylamide gel. Candidate insertion mutants were then relaxed and partially digested with *Bam*HI to determine the number of oligonucleotide linkers inserted.

A 100 bp synthetic palindrome used to create the palindromic insertion mutant pPAL-102 was generated by cleaving pUC-19 with *Eco*RI and then self-ligating the DNA at high concentration to promote concatemerization. The high molecular weight concatemeric DNA was then cleaved with *Sph*I and the digestion products were fractionated on a 5% non-denaturing polyacrylamide gel. A fragment corresponding in size to the 100 bp inverted repeat of pUC-19 polylinker sequence was excised as a gel slice, crushed and incubated in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 300 mM sodium acetate at 37°C for 16 hrs with constant agitation. The DNA was then separated from acrylamide fragments by filtration through glass wool and the purified DNA was precipitated with ethanol, washed with 70% ethanol and finally resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. This palindromic fragment was then blunt end ligated into the *Afl*II site of pSAD-2 to create pPAL-102 which contained a total insert size of 102 bp. pPAL-102 then served as the parent plasmid for the creation of a series of palindromic insertion mutants. Progressive removal of central axis sequence from pPAL-102 by digestion with a sequential series of restriction enzymes unique to the pUC-19 polylinker resulted in the generation of: pPAL-94 (*Kpn*I), pPAL-70 (*Sma*I), pPAL-48 (*Xba*I), and pPAL-40 (*Pst*I). The number of each pPAL clone refers to the size in base pairs of the palindrome inserted into the central axis *Afl*II site of pSAD-2 and the restriction enzyme that was used to delete the central axis sequence is bracketed following each clone.

Non-palindromic insertion mutants of pSAD-2 were generated in a manner similar to that used in the generation of linker insertion mutants. Restriction fragments generated by digestion of ØX174 replicative form DNA with either *Hinf*I or *Alu*I were blunt end ligated into the *Afl*II site of pSAD-2 The mutant clones resulting from insertion of *Alu*I fragments pDØA series; pDØA-28 (9 bp), pDØA-9 (42 bp), pDØA-2 (55 bp), pDØA-48 (75 bp) or of *Hinf*I fragments pDAH series; pDAH-30 (118 bp), were selected by ampicillin resistance and the insert size was determined by digestion with *Hind*III and *Eco*RI followed by fractionation of the digestion products on an 8% non-denaturing polyacrylamide gel. Insert sizes were later confirmed by DNA sequencing. A single arm of the pSAD-2 inverted repeat insert was cloned (pSDA-1) along with a direct repeat version (pSDA-46) by excising one arm of the inverted repeat as an *Afl*II-*Sst*I fragment and religating the digestion products under blunt end conditions. Candidate mutant plasmids were screened for insert size by polyacrylamide gel electrophoresis, and the structure of the inserts was confirmed by sequencing. A specific insertion mutant was generated using the procedure described above except that the heterologous sequence inserted was pre-defined as the 55 bp polylinker from pUC-19. This DNA was excised as a *Hind*III/ *Eco*RI fragment and the ends were blunted by incubation with T4 DNA polymerase and 250 μM dATP, dCTP, dTTP, and dGTP. The bluntended fragment was then ligated into the *Afl*II site of pSD-19 under blunt end conditions to create the insertion mutant pSD19-PLa. The presence of numerous well defined restriction sites within the insertion sequence made this construct particularly useful in an investigation of the structure of replicative intermediates.

Mutant versions of the cloned SFV replicative intermediate were generated in order to probe for the formation of heteroduplex DNA during the replication and resolution of these sequences. A site-specific mutation was introduced into the 1.5 kb inverted repeat insert of pSCB1a by first relaxing the plasmid to resorb all of the extruded cruciforms. Complete relaxation of pSCB1a required that the plasmid be incubated for 16 hrs on ice in 100 μg/ml ethidium bromide and in the absence of salt, during this incubation the DNA was protected from light to minimize nicking. Following the overnight incubation the DNA-ethidium bromide mixture was made to 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 0.5 mM DTT and extracted 6 times with ice cold, water saturated 2-N-butanol, followed by a single extraction with ice cold chloroform. Calf thymus topoisomerase I (2 U) was then added to the reaction and the tube was incubated in ice water which was slowly warmed to room temperature, 2 more units of topoisomerase were added and the reaction was allowed to proceed for 2 hrs at 37°C.The relaxed DNA was then extracted with phenol:chloroform (1:1) and chloroform and then precipitated with ethanol, washed with 70% ethanol and finally resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. This relaxed DNA was then subjected to partial digestion with restriction endonuclease *Xho*I and the resulting linear molecules were isolated by preparative gel electrophoresis. The purified linear molecules were incubated with phage T4 DNA polymerase in the appropriate buffer in the presence of 250 µM each of dTTP, dCTP, and dGTP. The resulting single base 5' overhang was removed by treatment with mungbean nuclease and the molecules were self-ligated under blunt end conditions. The resulting plasmid pSCBΔX contains a 2 bp insertion that destroys the *Xho*I site in one arm of the inverted repeat. This plasmid was used to test for the formation of heteroduplex DNA during the *in vivo* resolution process. BGMK cells (1 x 10^7) were infected with 2 ffu/cell of myxoma virus and were transfected with pSCB-1a or pSCBΔX. Following a 24 incubation period, the *in vivo* generated linear hairpin terminated minichromosome DNAs were harvested by a modification of the Hirt extraction procedure (Hirt, 1967). The infected cells were washed with 5 mls of cold phosphate buffered saline (PBS; 145 mM NaCl, 4 mM KCl, 10 mM phosphate, pH 7.3) + 5 mM EDTA followed by a wash with 5 mls of cold PBS. Cells were then lysed in the flasks by the addition of 1 ml: 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS and proteinase K (500 µg/ml). Following a 2 hr incubation at 37°C the cell homogenate was poured from the flasks into microfuge tubes and 250 µl of 5 M NaCl was added to each tube for a final concentration of 1 M NaCl. This suspension was mixed gently by inversion and then incubated on ice for 10 hrs. The precipitated high molecular weight DNA was then pelleted by centrifugation for 30 min at 12,000 rpm, 4°C in a Fisher micro-centrifuge. The supernatant fraction of this preparation was enriched for the low molecular weight covalently closed plasmid species. The DNA was further purified by 3 gentle extractions with an equal volume of buffered phenol, 1 extraction with an equal volume of phenol:chloroform (1:1), and 1 extraction with chloroform. The purified DNA was then concentrated by precipitation with 2 volumes of isopropanol and washed twice with 70% isopropanol. RNA was then removed by resuspending the DNA in 100 µl of 10 mM Tris-HCl pH 8.0 + 50 µg/ml RNase A, following which the sample was digested with *Dpn*I. The DNA was then purified by phenol:chloroform extraction and the linear monomer plasmid species were isolated by fractionation of the DNA through 1% low melting point agarose.

Inversions of the inverted repeat insert of SFV telomeric DNA were created by excising one arm of the inverted repeat of both pSD-48 and pSD-71 (previously described deletions of pSAIB 56a; DeLange *et al.*, 1986) as *Hind*III-*Afl*II fragments. Each purified fragment was then self-ligated to create concatemers and the high molecular weight DNA was cleaved with *Afl*II to create an *Afl*II-*Hind*III-*Afl*II inverted repeat. This dimer fragment was then isolated from the monomeric *Afl*II-*Hind*III fragment by polyacrylamide gel electrophoresis. The isolated fragments were eluted from the gel, purified, and blunt end ligated into the *Sma*I site of pUC-19 to create pSDI-48 and pSDI-71 which are inversions of the inverted repeat sequence contained in pSD-48 and pSD-71 respectively.

Linear hairpin terminated minichromosomes were generated *in vitro* from the circular supercoiled plasmids pSCB1a, pSAIB-56a and pSAD-2. The supercoiled parental plasmids contain large inverted repeats and exist as a series of topological isomers; a portion of these molecules extrude the inverted repeat insert into a cruciform configuration (DeLange *et al.*, 1986, Dickie *et al.*, 1987, Merchlinsky *et al.*, 1988). The base of the extruded cruciform is a four stranded DNA junction that is the structural equivalent of a Holliday junction recombination intermediate. These plasmids were cleaved with the phage T7 gene 3 endonuclease (purified from an overexpressing E. coli strain W3110 (pAR2510) by a modification of the procedure reported by deMassey *et al.*, (1987)). Cleavage across the base of the four stranded DNA junction yields a linear hairpin terminated plasmid DNA molecule with two ligateable nicks. The *in vitro* generated minichromosomes were ligated to seal the nicks and the linear molecules were then purified by preparative gel electrophoresis.

In vivo resolution assay. The *in vivo* transfection assay for the resolution of replicative intermediates has been described previously (DeLange & McFadden, 1986). Briefly; monolayers of SIRC $\cong 1 \times 10^6$ cells (80-90% confluent) in 35 mm wells of a 6 well multi-dish were infected with either SFV or Myxoma virus at a multiplicity of 1 focus forming unit (ffu)/cell in a volume of 200 µl. Following a one hour adsorption at 37°C, 2.5 mls of fresh DME/10% newborn calf serum was added to each monolayer. At this time 50-100 ng of plasmid DNA was precipitated in 200 µl of $CaPO_4$ in the absence of carrier DNA and the precipitate was applied directly to the monolayer. Following a 6 hr incubation the medium was aspirated and the monolayer was washed with PBS + 5 mM EDTA and then with PBS. Finally the infected/transfected monolayer was overlayed with fresh medium. Total DNA was harvested from the infected cells at 20-24 hrs post-transfection by lysis with 0.5% SDS, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 2 mg/ml proteinase K. After a 2 hr incubation at 37°C, the DNA was extracted sequentially with equal volumes of phenol, phenol:chloroform (1:1), and chloroform and then precipitated with ethanol, washed with 70% ethanol and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA. The resuspended DNA was then digested as indicated, fractionated on 0.7% agarose gels, transferred to nitrocellulose membranes (Bio-Rad Trans-Blot transfer medium) and probed with nick translated [^{32}P] labelled pUC-19 DNA. The hybridized blots were washed at high stringency and the labelled fragments were visualized by autoradiography. Resolution efficiency is qualitatively determined as a relative measure of the intensity of the linear monomer species compared to the amount of high molecular weight replicated DNA.

C. Results

Extensive *in vitro* mutagenesis has been performed on a cloned version of the SFV replicative intermediate in an attempt to define the properties of this sequence that are important for its resolution *in vivo*. Unidirectional deletion analysis of the cloned inverted repeat defined the regions of DNA sequence that form the essential cis-acting target sequence required for resolution of the replicative intermediate (DeLange *et al.*, 1986). Deletion of the 96 bp of non-coding sequence that forms the axis of dyad symmetry between the two inverted copies of the TRT had no deleterious effect upon the resolution of a cloned replicative intermediate. Thus the extrahelical bases that exist within the central axis region are not required for the resolution of the replicative intermediate and to date their function remains unknown. This observation also makes it apparent that any spatial requirements for the positioning of the inverted TRT sequences with regard to their ability to act as resolution targets are quite flexible.

The *in vivo* plasmid replication and resolution assay is schematically represented in Fig. II-1A. Poxvirus infected host cells will replicate any circular input plasmid DNA in a sequence nonspecific fashion. The replicated plasmid DNA sequences exist in the infected cells as high molecular weight concatemer structures (Fig. II-1B). Replication can be scored by resistance of the DNA to cleavage by *Dpn*I which will cleave the methylated input DNA but fails to cleave DNA that has been replicated in the poxvirus infected cells. This assay can be extended to investigate the resolution of poxvirus replicative intermediates by transfecting the infected cells with plasmid DNA substrates that contain a cloned version of a poxviral telomere replicative intermediate (Fig. II-1C). As is the case for any covalently closed plasmid DNA the cloned viral replicative intermediates are replicated to yield *Dpn*I resistant high molecular weight concatemers. A ladder of *Dpn*I resistant monomer and multimer, linear hairpin terminated minichromosomes are generated when poxvirus infected cells are transfected with plasmids that contain viral telomere replicative intermediates (Fig. II-1C).

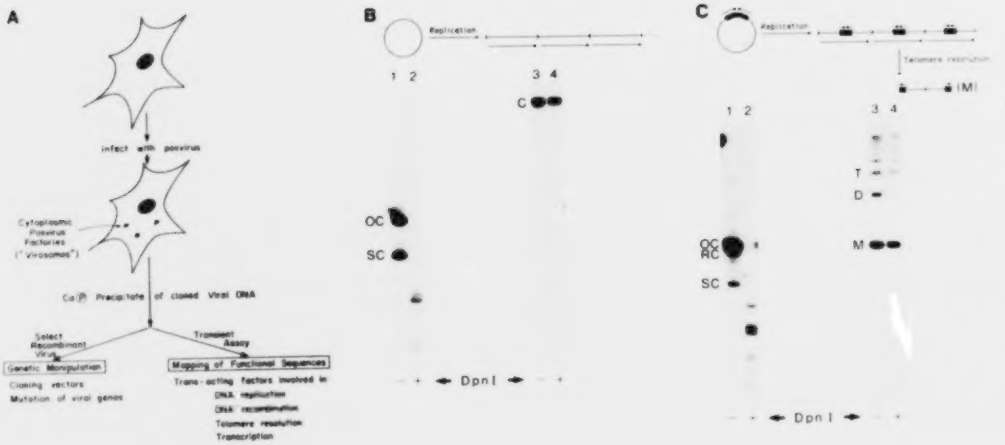
A
infect with poxvirus
Cytoplasmic Poxvirus Factories ("virosomes")
Ca(P) Precipitate of cloned Viral DNA
Select Recombinant Virus
Transient Assay
Genetic Manipulation
Cloning vectors
Mutation of viral genes
Mapping of Functional Sequences
Trans-acting factors involved in
DNA replication
DNA recombination
Telomere resolution
Transcription
B
Replication
1 2
3 4
C
OC
SC
- + Dpn I - +
C
Replication
Telomere resolution
(M)
1 2
3 4
T
D
M
OC
RC
SC
- + Dpn I - +

Figure II-1. The transfection assay for replication and telomere resolution in SFV infected cells. (A) Schematic representation of the protocol for calcium phosphate precipitation of cloned DNA for transient assay in the cytoplasm of poxvirus-infected cells. (B) Sequence nonspecific replication of plasmid DNA. Plasmid DNA containing a synthetic poly d(AT) : d(AT) inverted repeat, but no poxvirus sequences (pAT-34) was transfected into SFV infected SIRC cells, harvested at 3hr (prior to viral DNA replication; lanes 1, and 2) and 24 hr (after replication; lanes 3, and 4), digested with *Dpn*I or undigested and analyzed by Southern blotting, using vector DNA as probe. OC= open circular, SC= supercoiled, C= concatemers. (C) The conversion of transfected plasmid DNA containing the minimal core domain for SFV telomere resolution into linear minichromosomes. Conditions are as in panel B except the transfecting plasmid was pSAlB-56a, which contains 322 bp from the cloned SFV telomeric inverted repeat. Minichromosome sizes are indicated as M (monomer), D (dimer), T (trimer). RC=relaxed cruciform, which comigrates with OC during agarose gel electrophoresis.

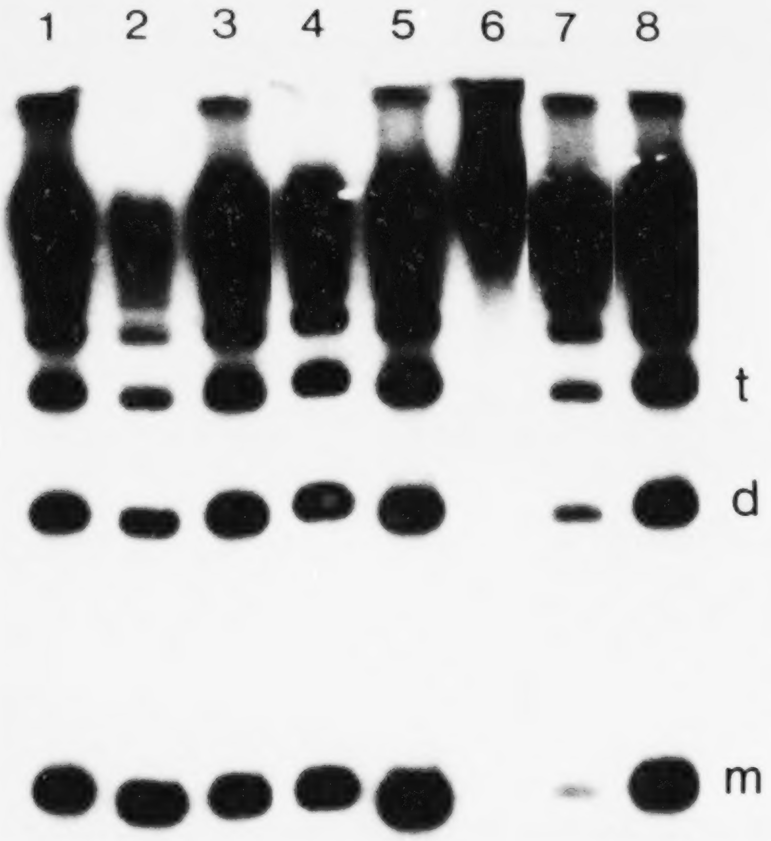

Figure II-2. Oligonucleotide linker insertion mutants of the cloned minimal core domain for SFV telomere resolution. The plasmids that carry mutant versions of the SFV telomere resolution domain were tested in the *in vivo* transfection assay for the ability to act as substrates for the viral resolution machinery. A Southern blot analysis of the resulting *Dpn*I resistant plasmid DNA is shown. The parental plasmid for the generation of the linker insertion mutants pSAD-2 (lane 5), negative control pUC-19 (lane 6), mutant versions of pSAD-2 containing 5 *Bam*HI linkers inserted into the central axis of the inverted repeat (lane 1), 1 linker (lane 2), 2 linkers, (lane 3), 4 linkers (lane 4). A deletion generated from an original version of the cloned SFV telomere replicative intermediate pSD-19 (lane 8) and a single linker insertion into the central axis of pSD-19 (lane 7). The sizes of the minichromosomes is indicated on the right of the figure monomers (m), dimers (d), trimers (t).

The effect of sequences intervening between the two inverted copies of the TRT was further investigated by the replacement of the native central axis sequence with multimers of a 10 bp double-stranded oligonucleotide linker. Figure II-2 demonstrates that the insertion of 5 (lane 1), 1 (lane 2), 2 (lane 3) or 4 (lane 4) copies of the oligonucleotide linker into the central axis of the inverted repeat of pSAD-2 had no detectable effect upon the replication and subsequent resolution of the plasmid when it was transfected into poxvirus infected cells. The replicative intermediate insert contained within pSAD-2 has 86 bp of the 96 bp of central axis sequence deleted, thus none of the insertions in figure II-2 caused an increase in the spacing between the inverted TRT sequences beyond that which occurs in the native replicative intermediate. Insertion of a single 10 bp oligonucleotide linker into the central axis of the replicative intermediate insert sequence of pSD-19 appears to have a negative effect upon the resolution of this plasmid *in vivo* but the resolution reaction is not eliminated (Fig. II-2, lane 7). All of the native central axis sequence is present in pSD-19 (Fig. II-2, lane 8) and in this plasmid the oligonucleotide linker has acted to increase the distance between the two TRT sequences. The insertional mutagenesis study was further extended by the use of a synthetic 100 bp perfect palindrome that was created from the polylinker sequence of pUC-19. This fragment was then used as the parent for the creation of a series of smaller perfect palindromes of 94 bp, 70 bp, 48 bp, 40 bp. Each of these fragments was blunt-end ligated into the *Afl*II site that forms the axis of dyad symmetry in the inverted repeat insert of pSAD-2. The family of plasmids derived from these insertions pPAL-102, pPAL-94, pPAL-70, pPAL-48, and pPAL-40 were tested for their ability to be replicated and resolved in an *in vivo* transfection assay. The results of this analysis are summarized in figure II-3. Palindromic insertion mutants are schematically illustrated (Fig. II-3A) pPAL-40, pPAL-48, pPAL-70, pPAL-94 and pPAL-102 are numbered 7 to 11 respectively. The size of the insertion is indicated within the box representing the inserted fragment. Insertion mutants pPAL-40, 48, 70 and 94 are replicated and resolved *in vivo* with efficiency greater than, or similar to that displayed by plasmids lacking insertions

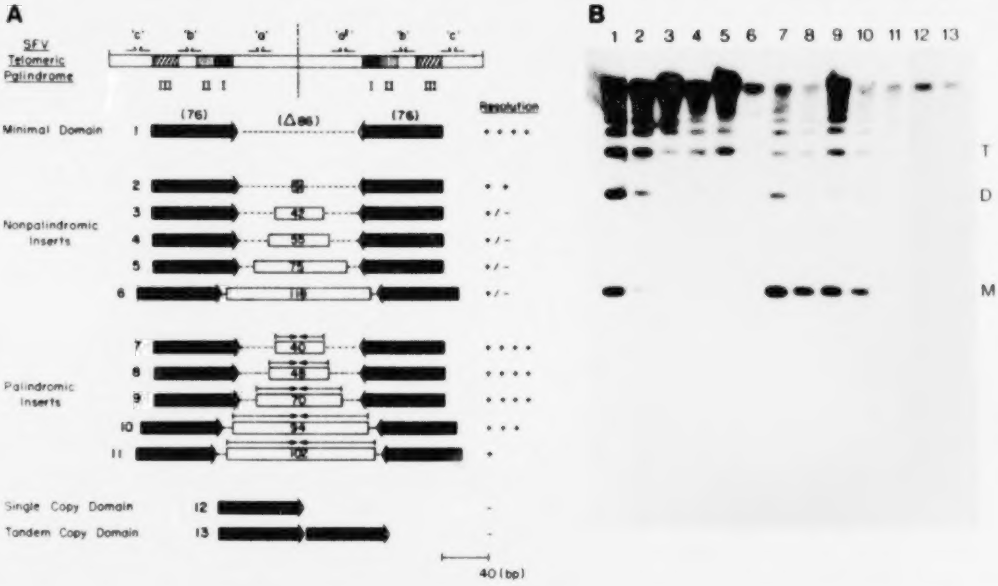
A
SFV
Telomeric
Palindrome
'c'
'b'
'a'
'a'
'b'
'c'
III
II
I
I
II
III
Resolution
(76)
(Δ86)
(76)
Minimal Domain
1
+ + + +
2
+ +
Nonpalindromic
Inserts
3
42
+/-
4
55
+/-
5
75
+/-
6
119
+/-
7
40
+ + + +
8
48
+ + + +
Palindromic
Inserts
9
70
+ + + +
10
94
+ + +
11
102
+
Single Copy Domain
12
-
Tandem Copy Domain
13
-
40 (bp)
B
1 2 3 4 5 6 7 8 9 10 11 12 13
T
D
M

Figure II-3. Effect of central axis symmetry variants on the resolution of the SFV telomere replicative intermediate. (A) variants of pSAD-2, which contains the minimal domain for SFV telomere resolution, were generated and tested for resolution into linear minichromosomes. 1. pSAD-2; 2. pDØA-28; 3. pDØA-9; 4. pDØA-2; 5. pDØA-48; 6. pDAH-30; 7. pPAL-40; 8. pPAL-48; 9. pPAL-70; 10. pPAL-94; 11. pPAL-102; pSDA-1; 13. pSDA-46. The resolution of plasmid species, indicated as + or - is a qualitative measure only (B) Southern blot of the resolved minichromosomes from panel A: monomers (M), dimers (D), trimers (T).

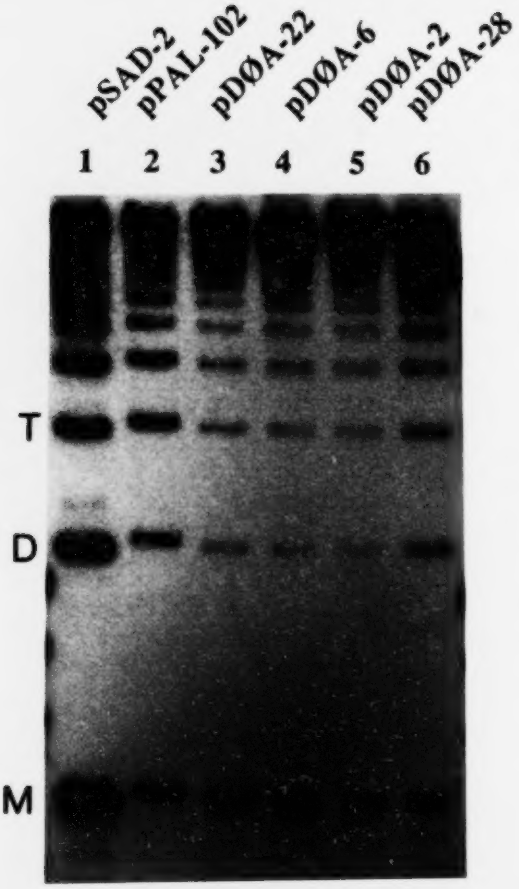

Figure II-4. The effect of palindromic and nonpalindromic insertions into the central axis of a cloned version of the minimal core sequence required for the *in vivo* resolution of an inverted repeat by poxviral *trans*-acting factors. All DNAs were analyzed by transfection into SFV infected cells and the DpnI resistant plasmid species were Southern blotted and probed with [^{32}P] labelled vector sequences. Lane 1. pSAD-2. Lane 2. pPAL-102. Lane 3. pDØA-22 , containing a 40 bp nonpalindromic insertion. Lane 4. pDØA-6, containing a 42 bp nonpalindromic insertion. Lane 5. pDØA-2, containing a 55 bp insertion. Lane 6. pDØA-28, containing a 9 bp insertion. Monomer (M), dimer (D), and trimer (T), linear minichromosomes are indicated at the right of the figure.

(Fig. II-3 B, lanes 7, 8, 9 and 10). A larger insertion mutant pPAL-102 is not resolved as efficiently *in vivo* (Fig. II-3B, lane 11) although with longer exposures some *Dpn*I resistant linear plasmid species can be detected (Fig. II-4, lane 2). Larger insertions of heterologous sequence are even more detrimental to the resolution process (M. Merchlinsky, personal communication). Resolution of the cloned SFV telomere replicative intermediates thus proceeds with wild type efficiency if the TRT sequences are separated by a total of 96 bp or less. Increasing the distance between the TRT domains results in a decrease in the resolution efficiency *in vivo* only when the two TRT sequences are separated by a greater distance than that which occurs in the native configuration.

All of the mutations described thus far have altered the distance between the inverted TRT sequences while maintaining the palindromic configuration of the replicative intermediate. The effect of interrupting the inverted repeat with a non-palindromic spacer is summarized in figure II-3. Constructs 2 to 6 consist of insertions of various sizes of ØX174 DNA into the central axis *Afl*II site of pSAD-2. The results of an *in vivo* transfection assay using the insertion mutants as substrates is shown (Fig. II-3 B). Small non-palindromic insertions of as little as 9 bp have a detrimental effect upon *in vivo* resolution (Fig. II-3 B, lane 2) and non-palindromic insertions of greater than 42 bp essentially abrogate the resolution reaction (Fig. II-3 B, lanes 3-6). These mutants therefore identify a requirement for palindromic DNA to intervene between the two TRT sequences.

The requirement for two inverted copies of the TRT sequence is demonstrated by transfection of infected cells with constructs that carry only a single copy of the TRT sequence or two copies of the TRT in a direct repeat tandem array (Fig. II-3 A, lanes 12 and 13). The replicated DNA from these transfections is recovered as high molecular weight structures with no detectable resolved species (Fig. II-3 B, lanes 12, and 13). To further test the requirement for a specific orientation of the two TRT sequences with respect to one another the arms of the palindrome were inverted with respect to the axis of symmetry such that the insert remained in a perfect inverted repeat configuration and the

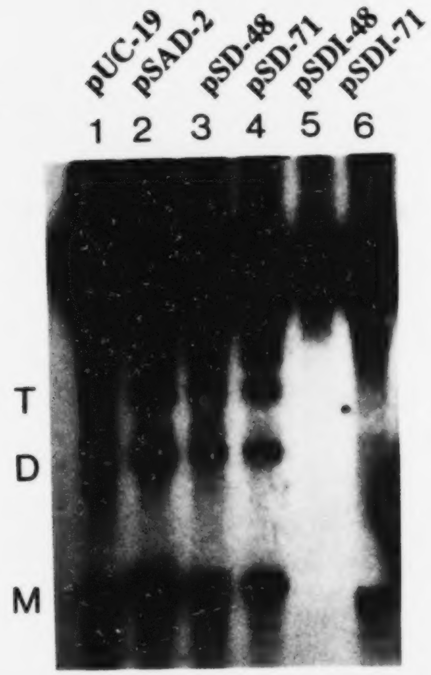
pUC-19
pSAD-2
pSD-48
pSD-71
pSDI-48
pSDI-71
1
2
3
4
5
6
T
D
M

Figure II-5. Inversion of the TRT sequences abrogates resolution *in vivo*. Lane 1 pSAD-2, lane 2 pUC-19. Deletion versions of the original SFV telomere replicative intermediate clone pSD-48 (lane 3) and pSD-71 (lane 4) are efficiently resolved *in vivo*. Inversions of replicative intermediates in these two plasmids pSDI-48 (lane 5) and pSDI-71 (lane 6) are not resolved. The positions of the monomer (M), dimer (D), and trimer (T) sized plasmids are indicated.

TRT sequences were separated by less than 98 nucleotide pairs. In these mutants the position of the TRT domains relative to one another have not been altered but the sequences are oriented opposite to the native configuration. *In vivo*, the inversion mutants are replicated but fail to resolve (Fig. II-5, lanes 5, and 6). Thus a requirement exists for the TRT sequences to be in a specific orientation to one another and to the axis of symmetry in order to function as substrates for resolution to minichromosomes.

In an effort to gain further insight into the mechanism of inverted repeat resolution in poxvirus infected cells a specific insertion mutant was generated and tested in the *in vivo* transfection assay. pSD19-PLa can not be resolved *in vivo* due to the nonpalindromic insertion present in the central axis. Although pSD19-PLa cannot assume a cruciform configuration it should be capable of undergoing homologous recombination and/or protein mediated DNA looping. It was hoped that a substrate of this nature could be used to trap intermediates in the resolution process and that the intermediates could be identified by altered mobility during agarose gel electrophoresis. pSD19-PLa is cleaved twice within the plasmid backbone by *Ava*II to yield fragments of 222 and 2799 bps (Fig. II-6, lane 1). The polylinker sequence inserted into the central axis of the inverted repeat replicative intermediate contains a unique *Xba*I restriction site and cleavage of pSD19-PLa with both *Ava*II and *Xba*I yields restriction fragments of 222, 997, and 1580 bps (Fig. II-6, lane 2) (Note that due to incomplete digestion, some 2799 bp fragment remains). When pSD19-PLa was transfected into SFV infected cells, the DNA that was recovered showed only the parental pattern when cleaved with *Ava*II (Fig. II-6, lanes 3, 4) or with *Ava*II and *Xba*I (Fig. II-6, lanes 5, 6). No higher molecular weight structures indicative of strand-exchange or recombination intermediates could be identified even after long over exposures of the gel (Fig. II-6, lanes 7, 8, and 9).

Plasmid constructs that contain large inverted repeat sequences like pSCB-1a and its derivative pSCBΔX can assume either a line form or a cruciform configuration (Fig. II-7). The *Xho*I restriction sites in the pSCB-1a insert are symmetrically arranged and so when

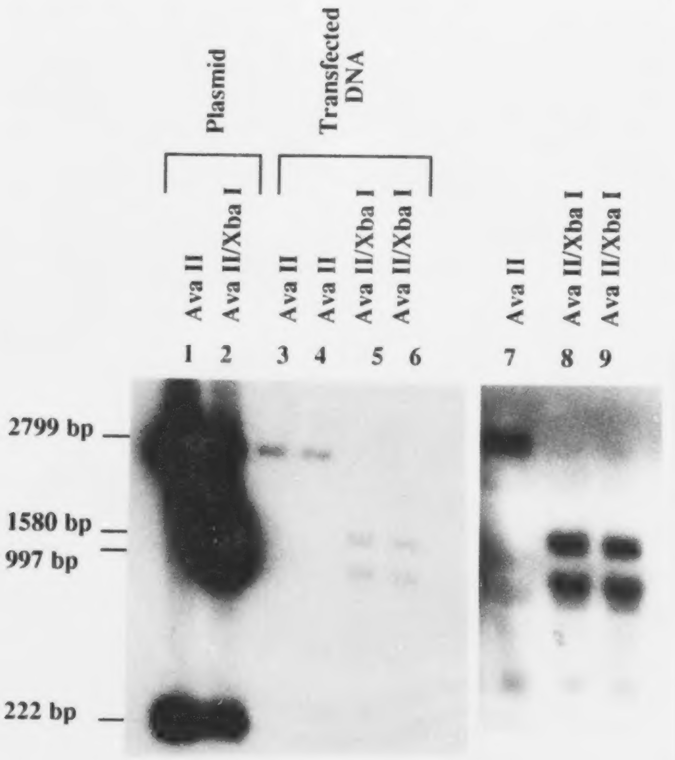
Plasmid
Transfected DNA
Ava II
Ava II/Xba I
Ava II
Ava II
Ava II/Xba I
Ava II/Xba I
Ava II
Ava II/Xba I
Ava II/Xba I
1
2
3
4
5
6
7
8
9
2799 bp
1580 bp
997 bp
222 bp

Figure II-6. Southern blot analysis of a plasmid designed to trap strand-exchanged resolution intermediates. pSD19-PLa was transfected into SFV infected SIRC cells and the *Dpn*I resistant DNA was recovered and cleaved with *Ava*II (lanes 3, and 4) or *Ava*II and *Xba*I (lanes 5, and 6). The substrate plasmid cleaved with *Ava*II (lane 1) or *Ava*II plus *Xba*I (lane 2) was used as a marker. The gel was greatly overexposed to detect any small portion of the DNA that might represent trapped intermediates (lanes 7, 8, and 9).

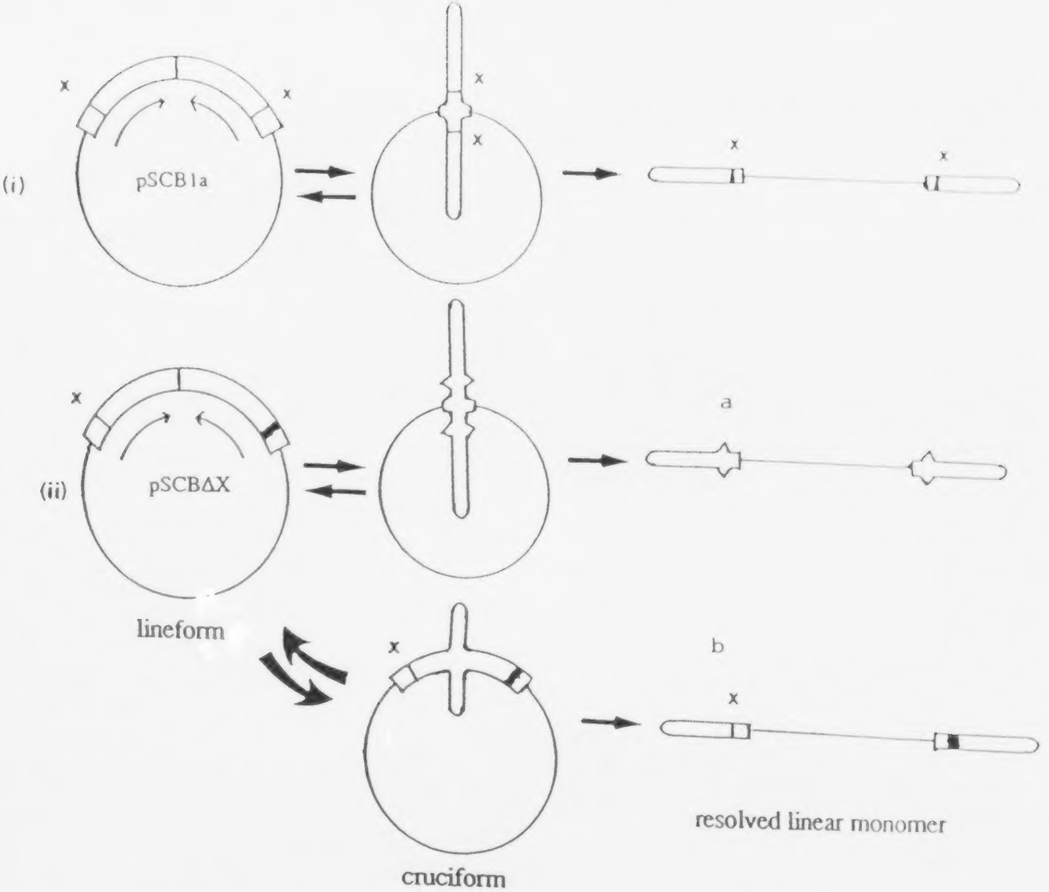
(i)
x
x
pSCB1a
x
x
x
x
(ii)
x
pSCBΔX
a
lineform
x
b
x
resolved linear monomer
cruciform

Figure II-7. Predictions of the cruciform resolution model. The inverted repeat insert is extruded to a great extent *in vitro* such that in a portion of the molecules the symmetric *Xho*I sites (x) are present on the arms of an extruded cruciform. Resolution of this molecule *in vivo* has no effect upon the *Xho*I sites. *In vitro* some molecules of pSCBΔX which has one *Xho*I site destroyed by mutagenesis, extrude the inverted repeat such that both of the *Xho*I sites are destroyed by the formation of heteroduplex DNA (iia). A resolution mechanism that requires only limited cruciform extrusion retains the integrity of the single *Xho*I site (iib).

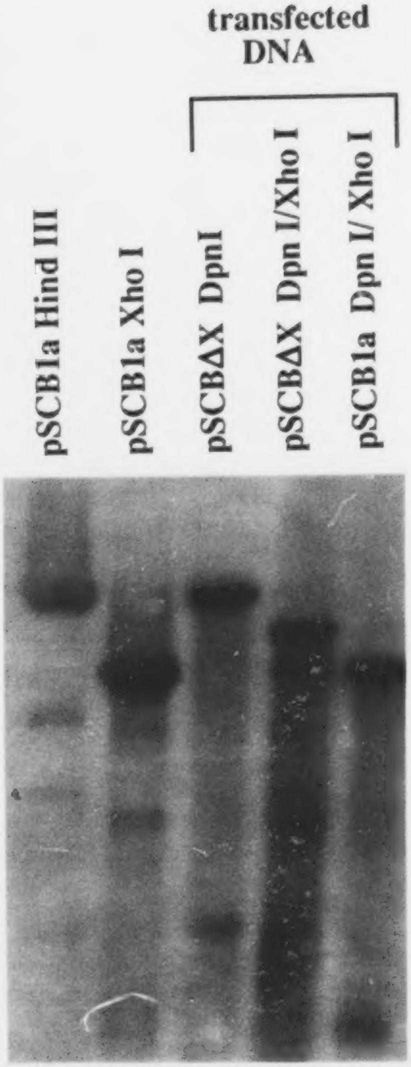
transfected DNA
pSCB1a Hind III
pSCB1a Xho I
pSCBΔX DpnI
pSCBΔX Dpn I/Xho I
pSCB1a Dpn I/ Xho I

Figure II-8. Southern blot analysis of transfected pSCB1a and pSCBΔX. Plasmid DNA pSCB1a cleaved with *Hind*III (linear) or *Xho*I was used as a marker. Linear *Dpn*I resistant plasmid minichromosomes resolved *in vivo* were analyzed by Southern blotting without prior cleavage, or following cleavage with *Xho*I.

this plasmid is resolved into a linear minichromosome the *Xho*I sites are retained. The *Xho*I site in pSCBΔX is unique and so any mechanism of resolution that involves strand-exchange past this site in the inverted repeat will result in a linear minichromosome that lacks any *Xho*I sites (Fig. II-7 iia). On the other hand if resolution requires only the sharing of strands very near the axis of symmetry, the unique *Xho*I site will remain intact (Fig. II-7, iib). When pSCBΔX linear minichromosomes were extracted from cells that had been infected with Myxoma virus, the DNA uniformly displayed a restriction pattern indicative of the presence of one intact *Xho*I site (Fig. II-8, lane 4). Thus, resolution of pSCBΔX did not result in the formation of heteroduplex DNA in the region of the *Xho*I site.

The ladder of linear monomer and multimer hairpin terminated minichromosomes generated by the replication and resolution of transfected plasmid DNA consists of covalently closed molecules that rapidly reanneal following thermal denaturation (Fig. II-9). Palindromic insertion mutants are efficiently resolved *in vivo* and rapidly reanneal following denaturation (Fig. II-9, lanes 1-6) as do the linear products generated by a plasmid carrying a wild type SFV replicative intermediate. Insertion mutants in which the inverted repeat is disrupted with a non-palindromic sequence are resolved poorly , pDØA-9 (lane 7), pDAH-30 (lane 9) and pDØA-48 (lane 11). A large portion of the DNA produced by transfection of these non-palindromic insertion mutants into infected cells is not covalently closed and appears as a smear following thermal denaturation and quick chilling (Fig. II-9, lanes 8, 10, and 12).

All of the mutant analysis thus far described was performed using circular plasmid substrates in an *in vivo* transfection assay. In an effort to develop a model that might more accurately represent viral replication and resolution processes, a series of linear plasmid DNAs with viral hairpin telomere sequences that mimicked the viral genome structure were constructed. Linear hairpin terminated plasmids were generated by using phage T7 gene 3 endonuclease to cleave circular plasmids that were extruding their inverted repeat inserts as

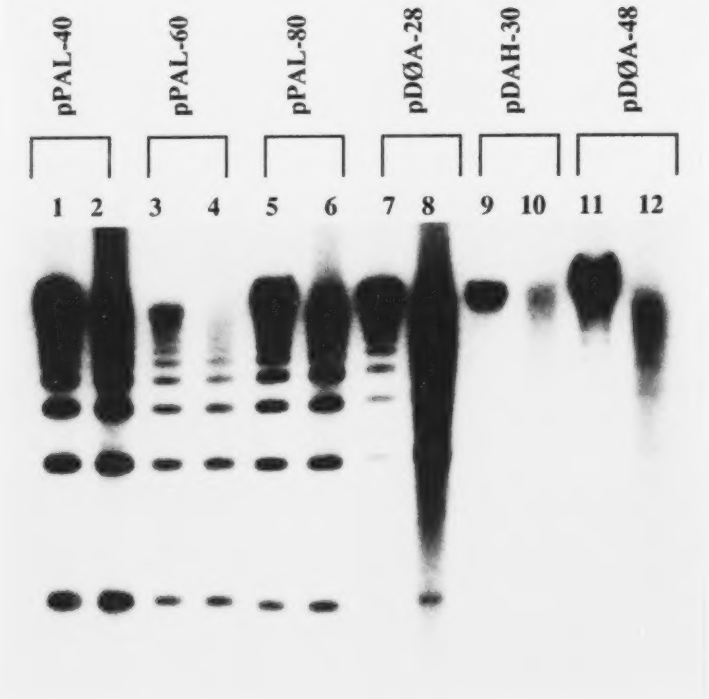
pPAL-40
pPAL-60
pPAL-80
pDØA-28
pDAH-30
pDØA-48
1
2
3
4
5
6
7
8
9
10
11
12

Figure II-9. Analysis of the covalently closed nature of plasmid constructs that were replicated and resolved *in vivo*. Plasmid derivatives of pSAD-2 that contain palindromic insertions (pPAL-40, pPAL- 60, pPAL 80) or nonpalindromic insertions (pDØA-28, pDAH-30, pDØA-48) were transfected into SFV infected cells and the *Dpn*I resistant DNA in lanes 2, 4, 6, 8, 10, and 12 was boiled for 5 min before quick-chilling and Southern blot analysis. A duplicate of each sample was electrophoresed in parallel without boiling and quick-chilling (lanes 1, 3, 5, 7, 9, 11).

cruciforms. The T7 gene 3 endonuclease recognizes the base of an extruded cruciform as a Holliday junction analog, and by nicking opposing strands the four-way junction is resolved yielding a linear hairpin terminated DNA molecule (deMassey *et al.*, 1987). The *in vitro* resolved plasmids were then ligated, purified by preparative gel electrophoresis and used as substrates in an *in vivo* resolution assay. All of the linear minichromosomes that possess intact copies of the TRT sequence are replicated and resolved *in vivo* to create a ladder of minichromosomes that is indistinguishable from the products generated when a circular substrate is used (Fig. II-10). The linear products are all resistant to restriction endonuclease *Dpn*I which cleaves only the unreplicated methylated input DNA (Fig. II-10, lanes 1, 3, and 5), but are all sensitive to the *Mbo*I which cleaves only the replicated DNA (Fig. II-10, lanes 2, 4, and 6). Thus in spite of the greater resemblance to the viral genome the linear plasmid substrates appear to offer no advantage over the circular plasmids in that they are resolved with similar efficiency.

During the course of these experiments it has been observed that the extrahelical base composition of the inverted repeat inserts is stably maintained in E. coli strain DB1256 which is defective in homologous recombination but is competent to perform mismatch repair. While the inverted repeat insert is in a lineform configuration all of the nucleotides in the construct can be expected to be basepaired. If the inverted repeat is extruded into a cruciform structure the asymmetric base mismatches would be exposed and subject to mismatch repair. Since we do not observe a detectable level of loss of the extrahelical bases within the full size construct we presume that *in vivo* either the inverted repeat is not extruded as a cruciform or that extruded cruciforms are rapidly resolved and removed from the pool of replicating DNA. In the latter case the substrate molecules that reveal targets for mismatch repair also expose themselves as recombination intermediates that are resolved by the host enzymes.

D. Discussion

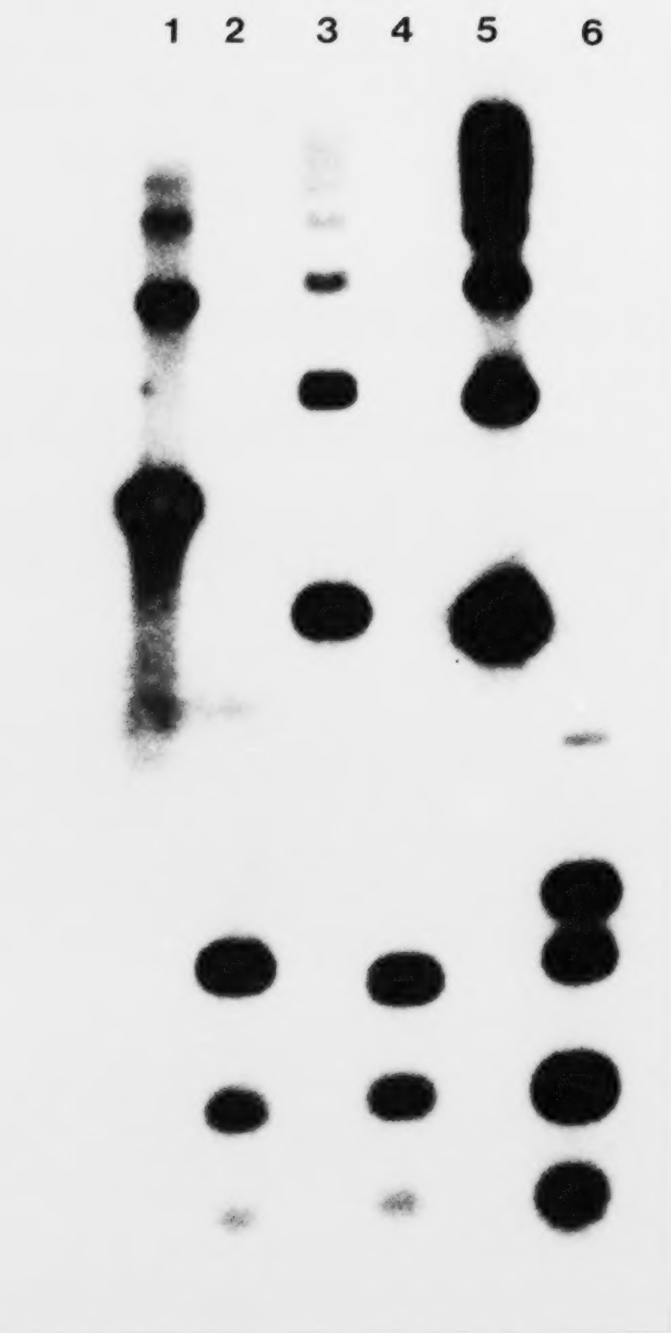

Figure II-10. *In vivo* replication and resolution of linear hairpin terminated minichromosomes generated *in vitro*. Linear minichromosome versions of pSCB1a (lanes 1, and 2), pSAlB-56a (lanes 3, and 4), and pSAD-2 (lanes 5, and 6) were transfected into SFV infected cells and the recovered DNA was digested with *Dpn*I to degrade unreplicated input DNA (lanes 1, 3, and 5), or with *Mbo*I to degrade all of the replicated DNA species (lanes 2, 4, and 6).

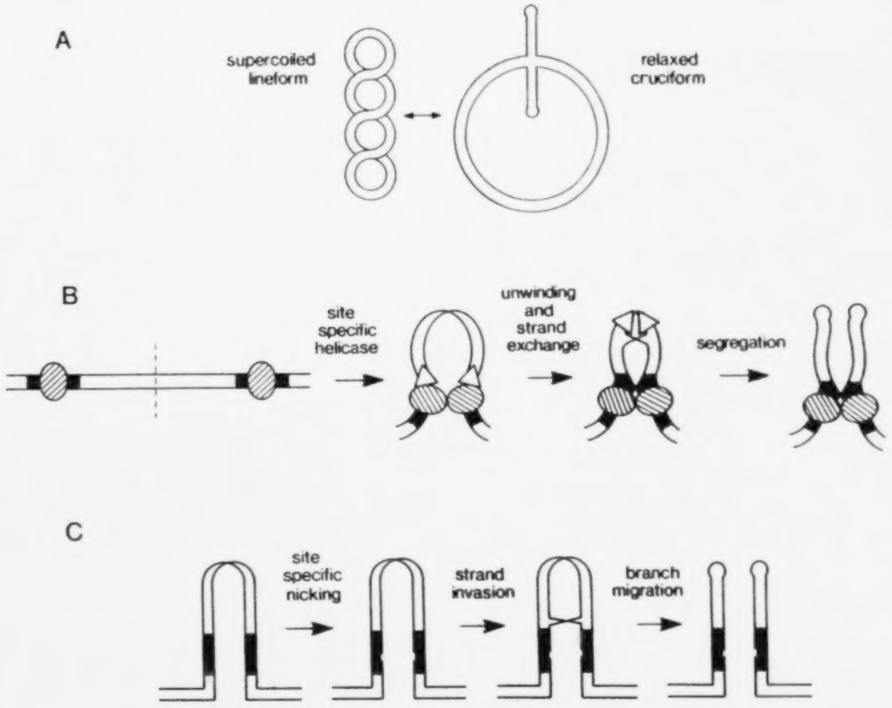
A
supercoiled lineform
relaxed cruciform
B
site specific helicase
unwinding and strand exchange
segregation
C
site specific nicking
strand invasion
branch migration

Figure II-11. Possible models for poxvirus telomere replicative intermediate resolution. (A) Torsional strain induced by negative supercoiling can induce and stabilize the cruciform configuration of an inverted repeat *in vitro*. A structure of this nature forms a target for Holliday junction resolving enzymes but it is not clear whether this type of structure can be induced to form *in vivo*. (B) Symmetrical binding by viral proteins to the two inverted copies of the TRT could result in unwinding of the TRT helix by an orientation specific helicase with subsequent rewinding and the formation of a recombination intermediate that would subsequently be resolved. (C) protein mediated DNA looping followed by strand exchange and directed branch migration could potentially be initiated in a sequence specific fashion and the resulting interwound structures could be unlinked by the virally encoded type I topoisomerase.

Directed *in vitro* mutagenesis has been utilized to investigate the significance of DNA sequence organization and structure in the resolution of poxvirus replicative intermediates. This approach has been extended to develop insight in to the mechanism of replicative intermediate resolution. In this investigation extensive use has been made of the observation that cloned inverted repeat versions of poxvirus telomeres are replicated and subsequently resolved into hairpin terminated linear minichromosomes when transfected into poxvirus infected cells (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). In most respects (particularly sequence dependence and the structure of the resolved products) this transient assay appears to mimic the process by which hairpin terminated progeny virus are produced. Thus, we presume that the conclusions drawn from the resolution of cloned viral substrates can be accurately applied to the resolution of viral replicative intermediates. Linear plasmid minichromosomes with viral hairpin termini that have been generated *in vitro* mimic the structure of the mature viral genome more closely than do the circular plasmid substrates. The linear minichromosomes are replicated and resolved to create a ladder of minichromosome monomers and multimers identical to that created by circular plasmids when transfected into poxvirus infected cells. This suggests that during replication the *in vitro* generated minichromosomes may transiently pass through the same types of intermediate structures as do the circular plasmids. The topological state of resolution competent substrates *in vivo* has not been determined and it is not clear if the circular plasmids can act as substrates in the resolution reaction or if it is first necessary for them to be replicated into linear concatemer structures. Thus we can only conclude that the common intermediate structure most likely involves an inverted repeat of the replicated hairpin sequences.

The sequence non-specific replication of plasmid DNA in poxvirus infected cells suggests that DNA synthesis in poxvirus infected cell cytoplasms may be a promiscuous event and that the viral replicative machinery utilizes any available DNA as a template. The existence of a specific origin of replication for poxvirus DNA can not be formally excluded;

however, to date no rigorous evidence has been presented to indicate that viral DNA synthesis initiates at a specific sequence on the viral genome. The particular details of how the poxvirus genome is replicated remain obscure; however, it is clear that replication through the hairpin termini transiently produces inverted repeat structures. Since the overall topological state of the replicating viral DNA is unknown, the context in which the telomere inverted repeat exists is not certain. It is possible that the palindromic replicative intermediate exists not only as the junction between multimers of nascent viral genomes but could also be incorporated in a terminal replication bubble, or in circular replicative intermediates. The existence of linear concatemers of replicating viral DNA has been demonstrated by pulsed-field gel electrophoresis (DeLange, 1989, Merchlinsky & Moss, 1989a). One interesting contrast between the replicating plasmids and viral DNA is that a relatively small portion of the plasmid DNA is resolved *in vivo*, and most of the plasmid sequence can be detected as a ladder of multimers. The replicating viral DNA is primarily detected as very high molecular weight structures and as linear monomers. Relatively little intermediate sized viral DNA is ever detected during the replication of a wild type virus; however, numerous thermolabile strains of vaccinia virus that are deficient in the resolution of replicative intermediates due to defects in late protein synthesis display a ladder of multimer DNA species during replication at the non-permissive temperature (DeLange, 1989, Merchlinsky & Moss, 1989a). One interpretation of this observation is that replication and resolution of the plasmids is a relatively inefficient process due to the sequestration of viral gene products into "virosomes" to which plasmid DNAs may have limited access. Plasmid DNA is replicated and resolved most efficiently when transfected into the infected cells at an early time in the infective cycle. Transfecting plasmid DNA into infected cells at late times during the infection results in much less replicated and resolved plasmid sequences. It is not certain whether this is because the infected cells take up DNA less readily or because the DNA taken up can not gain access to the viral replicative machinery. The ladder of multimer minichromosomes produced by resolution of replicated plasmid DNA can not be chased into a monomer form. This was determined by allowing the infection to proceed for greater lengths of time or by blocking DNA replication after resolution has been initiated. The stability of the plasmid multimers seen in the transfection assay, suggests that these species may be dead end intermediates that can no longer function as substrates for resolution. It could also be that the ladder of minichromosomes is maintained in an equilibrium state largely by homologous recombination among the plasmid DNAs. Plasmid multimers generated *in vivo* can be isolated and purified by preparative gel electrophoresis and then re-transfected into poxvirus infected cells where they are replicated and resolved to create a complete ladder of monomers, dimers, and trimers all the way up to very high molecular weight DNA, suggesting that these structures are not dead end molecules but are merely intermediates in the resolution or recombination pathways (data not shown).

It has been reported that much of the replicating poxvirus genomic DNA exists as nicked intermediate structures (Holowczak & Diamond, 1976). The linear minichromosomes produced by the replication and resolution of circular plasmids containing inverted repeat viral replicative intermediates appear to rapidly reanneal following thermal denaturation suggesting that they consist of covalently closed structures. Palindromic insertions into the central axis of the inverted repeat have little effect upon the production of covalently closed linear minichromosomes (Fig. II-9, lanes 1-6); however, non-palindromic insertions result in the production of a considerable amount of minichromosome DNA that fails to reanneal following denaturation (Fig. II-9, lanes 7-12). This result is consistent with the non-palindromic insertions having a disruptive effect upon a later stage in the resolution process rather than on an initial event. For example, DNA could be nicked and strand-exchange could initiate but could not be completed. This would lead to the accumulation of nicked intermediate structures that would be sensitive to denaturation.

The sequence independence of plasmid DNA replication in poxvirus infected cells is in contrast to the requirement for distinct sequences to catalyze the resolution of viral replicative intermediates. The target sequences required for telomere resolution have been well defined by both deletion analysis and and site-specific mutagenesis (DeLange *et al.*, 1986, DeLange & McFadden, 1987, Merchlinsky & Moss, 1989b, Merchlinsky, 1990b). Almost all of the 96 bp of sequence including the extrahelical bases that separate the two inverted TRT domains can be deleted from the cloned SFV telomere replicative intermediate with no deleterious effect upon the ability of this plasmid to act as a substrate for resolution *in vivo* (DeLange & McFadden, 1987). This observation indicates that the central axis sequences are not essential for resolution but they may play some important role that can not be detected by this assay, for example packaging of the nascent viral genomes. The deletion of central axis sequences indicates that the spacing requirements between the two resolution domains is quite flexible. The ability to introduce various lengths of heterologous palindromic DNA into the symmetry axis between the two inverted copies of the TRT sequence shows first that the nucleotide sequence of the spacer is not important for telomere resolution. This also indicates that there is no "side of helix effect" as has been shown to occur when protein mediated DNA looping occurs through the interaction of proteins that bind in a sequence specific fashion to only one side of a DNA helix as in the case of the E. coli Ara operon (Schleif, 1987). All of the palindromic insertion mutants functioned as suitable substrates for the viral *trans*-acting factors that catalyze the resolution of replicative intermediates. However, when palindromic sequences longer than the native sequence that intervenes between the TRTs in the replicative intermediate were inserted into the symmetry axis of pSAD-2, a decrease in resolution efficiency occurred (Fig. II-2 lane 7, Fig. II-3 lane 11, Fig. II-4, lane 2). These mutants therefore identify a requirement for perfect or near perfect inverted repeat symmetry within the replicative intermediate structure. The requirement for inverted repeat symmetry can be interpreted as an indication that homologous sequences are required, possibly for strand-exchange and branch migration between the two sides of the inverted repeat. This interpretation is in fact the only way to reconcile the position of the extrahelical bases in the hairpins that result from the resolution of the inverted repeat. The requirement for palindromic DNA between the TRT sequences is consistent with a model where strand-exchange nucleates at the axis of symmetry and then branch migration leads to the extrusion of a cruciform structure (Fig. II-11 A). A similar model invokes DNA looping with protein mediated recombination and strand-exchange occurring at the TRT followed by branch migration directed toward the axis of symmetry (Fig II-11 B). Interruption of the dyad symmetry axis with a non-palindromic insertion is lethal to the resolution of cloned SFV replicative intermediates. This result would be predicted by either of the two models previously described because in the first case the insertion would prevent the nucleation of a cruciform at the axis of symmetry or would at least cause a considerable increase in the energy required to maintain a stably extruded cruciform. On the other hand DNA looping and recombination at the TRT should not be prevented by a non-palindromic insertion but branch migration would be limited and the unlinking event required at the central axis would be prevented. It was predicted that if strand-exchange were initiated between the TRT sequences followed by branch migration directed toward the axis of symmetry the crossover intermediate generated would be trapped by a nonpalindromic insertion within the central axis of the inverted repeat. Cleavage of this intermediate structure within the unique sequences of the nonpalindromic insertion and within the pUC vector sequences would reveal a linked intermediate structure that migrates more slowly than the products generated by cleaving the parental plasmid. No such trapped intermediates could be detected by Southern blotting plasmid DNA that had been extracted from SFV infected cells (Fig. II-6). Thus it is possible that resolution does not proceed through this type of strand-exchange intermediate; however, a more trivial explanation is that the intermediate crossover structure is very labile and susceptible to breakage or nicking during the extraction procedure. The distance effect revealed by the large palindromic insertions is more difficult to reconcile with either of the postulated models for telomere resolution. The small increase in spacing introduced between the TRT sequences in this study are predicted to have little if any effect upon protein mediated DNA looping and recombination or helicase activity at the TRT sequences (Fig. II-11 B, and II-11 C). A constraint upon this model that can be envisaged is that the intermediate formed by looping and recombination is topologically isolated from the rest of the molecule, in order to branch migrate toward the axis of symmetry one turn of helix must be relaxed for each turn that the migrating strands advance. The virally encoded topoisomerase could potentially serve to relax the loop ahead of the migrating strands. Increasing the length of DNA between the TRT sequences would increase the number of turns of helix to be relaxed prior to segregation of the strands as hairpin structures and this could prove to be a bottle neck to resolution. Branch migration driven by superhelical torsion and proceeding through a cruciform intermediate (Fig II-11 A) is not predicted to be limited by palindromic insertions, in fact, plasmids that contain large inverted repeats of SFV telomeric sequence readily extrude these sequences under relatively mild superhelical tension *in vitro* (Dickie *et al*., 1987). There is very little kinetic constraint to the extrusion of most or all of the inverted repeat sequence *in vitro*. If cruciformation were driven in a similar fashion *in vivo*, a population of molecules with various lengths of extruded cruciform would be predicted to exist. We have been unable to detect the formation of heteroduplex DNA proximal to the TRT sequence in a replicated and resolved substrate plasmid DNA. One interpretation of this data is that if cruciform extrusion does occur *in vivo* it is limited to the region of sequence between the TRTs and the axis of symmetry. There is at present no evidence that cruciform structures are extruded in poxvirus infected cells; however, cruciform binding proteins have been isolated from eukaryotic cells (Elborough & West, 1988) and it is possible that sequence specific poxvirally encoded proteins bind to the replicative intermediate structure and induce the extrusion of a cruciform that could subsequently be resolved by either viral or host cell nucleases. Increasing the distance between the two binding sites might be detrimental by limiting the ability of viral proteins to drive cruciformation. If simple unwinding of the DNA helix at the TRT sequence allows the formation of a secondary structure that acts as a target for resolving activities then moving the TRT sites apart might act to limit the formation of secondary structure without affecting binding and unwinding at the TRT.

A strict requirement for two inverted copies of the TRT sequence has been demonstrated by the failure of a single copy or a tandem direct repeat version of the TRT sequences to be resolved when transfected into poxvirus infected cells. The requirement for two copies of the TRT sequence implies the occurrence of symmetrical events such as protein binding and unwinding or some form of communication between the two sites such as would be expected from a site-specific recombination event. A direct repeat of the TRT sequence destroys the palindromic nature of the replicative intermediate thus preventing strand-exchange and potentially limiting the ability of the TRT sequences to be aligned for the initiation of a site-specific recombination event. In this investigation the tandem repeat of the TRT sequence was generated by flipping the entire arm of the inverted repeat so that the position of the TRT sequence was unchanged with respect to flanking sequences that may influence resolution. In another investigation, only the 20 bp TRT sequences were flipped while the flanking sequences were unaltered thus maintaining the palindromic nature of the sequences intervening between the TRTs (Merchlinsky, 1990a). In both of these cases the mutated version of the poxvirus replicative intermediate was replicated but failed to be resolved by the viral *trans*-acting factors. The altered orientation of the TRT domains creates two potential problems: (1) If sequence-specific and strand-specific nicking at the TRT is involved in resolution, then a tandem repeat may present a target for the initial nicking event, but instead of creating nicks on opposite strands on either side of the symmetry axis, the nicks would be created uniquely in only one strand. (2) Similarly, if an orientation specific helicase is involved in helix unwinding and directed branch migration then a tandem copy of the TRT would lead to misdirection of the helicase activity away from the axis between the two TRT sequences.

We have inverted the orientation of the two TRT sequences with respect to the symmetry axis in a cloned SFV telomere replicative intermediate. This maintains the palindromic nature of the sequence intervening between the TRTs and the complementarity of the TRT sequences on the opposite sides of the symmetry axis. Strand-specific nicking and refolding should not have been prevented by this manipulation. The ability of the TRT sequences to loop together in a protein mediated fashion also remained intact. One activity that would be abrogated by inversion of the TRT sequences is strand-exchange and branch migration directed by an orientation specific helicase. Misdirection of the helicase activity with the corresponding loss of the ability to correctly segregate the heteroduplex DNA would explain the inability of the inverted TRTs to act as resolution substrates.

The data presented in this report identify three requirements for the resolution of poxvirus replicative intermediates. Requirements for a specific orientation of the TRT sequences, an effect of the distance between the two inverted TRT domains, and palindromic sequences between the TRT domains have been demonstrated. The elucidation of these requirements has allowed the initial development of several models that may explain the mechanisms of hairpin telomere maintenance. Further clarification of the mechanism of telomere resolution will be greatly aided by the analysis of conditional lethal poxvirus variants that are specifically defective in telomere resolution and by the development of a biochemically defined *in vitro* system for the resolution of telomere replicative intermediates.

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Chapter III

The Target DNA Sequence for Resolution of Poxvirus Replicative Intermediates is an Active Late Promoter

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A. Introduction

Linear DNA genomes are a common feature of complex eukaryotic organisms and many viruses (Berns, 1990, Kelly *et al.*, 1988). The complete and accurate replication of linear DNA molecules requires some special mechanism to account for the need of all known DNA polymerases to utilize a 3'-hydroxyl to prime synthesis (Cavalier-Smith, 1974, Watson, 1972). Many eukaryotic organisms have overcome the problem of replicating chromosome termini by the use of a telomere terminal transferase (telomerase) activity, which synthesizes terminal sequence in a template independent fashion (reviewed in Blackburn, 1990, Zakian, 1989). An alternative strategy for the replication and maintenance of chromosome termini is the possession of covalently linked terminal hairpin loops. This type of DNA structure has been identified in a variety of eukaryotic organelle and plasmid chromosomes (Blackburn & Gall, 1978, Ellis & Day, 1986, Pritchard & Cummings, 1981), parvoviruses (Berns, 1990), African swine fever virus (Gonzales *et al.*, 1986) and poxviruses (Baroudy *et al.*, 1982, DeLange *et al.*, 1986). The Leporipoxvirus Shope fibroma virus (SFV) and Orthopoxvirus vaccinia virus have double stranded linear DNA genomes with hairpin termini which exist in "flip" and "flop" configurations (Baroudy *et al.*, 1982, DeLange *et al.*, 1986). During the viral replicative cycle DNA synthesis through the hairpin loops transiently generates inverted repeat structures (Moyer & Graves, 1981) which fuse nascent viral genomes together in arrays of linear concatemers (DeLange, 1989, Merchlinsky & Moss, 1989). Production of mature progeny viral DNA requires that the inverted repeat replicative intermediates be resolved to yield unit length genomes with "flip" and "flop" hairpin termini. The cytoplasmic site of viral replication and the ability of poxviruses to replicate in enucleated cells (Moss, 1990) suggest that all of the activities required for telomere replication and resolution are virally encoded (reviewed in DeLange & McFadden, 1990).

Investigations into the resolution process have been greatly aided by the observation that cloned versions of the telomere replicative intermediates are replicated and resolved into linear minichromosomes with viral hairpin termini when transfected into virus infected cells (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). The minimal viral DNA sequences required in *cis* for the resolution of replicative intermediates have been determined by both deletion and point mutation analysis (DeLange *et al.*, 1986, DeLange & McFadden, 1987, Merchlinsky & Moss, 1989a) to be a stretch of about 20 nucleotides located near the hairpin termini. This target sequence for the viral resolution machinery has been designated the telomere resolution target (TRT) (DeLange & McFadden, 1990). Despite the overall lack of sequence homology near the termini between different genera of poxviruses for which this information is available, the deduced TRT amongst various poxviruses shows remarkable conservation (DeLange & McFadden, 1990) suggesting that a common resolution mechanism may be utilized by all poxviruses. This is underscored by the fact that SFV and vaccinia virus can resolve each others telomeres (DeLange *et al.*, 1986) and is consistent with the observation that these viruses recognize each others promoters (Macaulay & McFadden, 1989) and early transcription termination signals (Upton, *et al.*, 1987, Yuen & Moss, 1987).

Inspection of the conserved minimal TRT sequence reveals that it is very similar to the recently defined consensus sequence for poxvirus late promoters (Davison & Moss, 1989). This sequence similarity and the recent report that the telomeres of the Orthopoxvirus vaccinia virus are transcribed at late times during infection (Parsons & Pickup, 1990) suggests the possibility that transcriptional events may in some way influence DNA replication or telomere resolution.

In order to study the possible role of transcription in telomere resolution we have undertaken an investigation of the ability of the SFV TRT to function as a viral promoter. In this communication we report the existence of an intrinsic relationship between telomere resolution and specific late promoter activity. Potential mechanisms by which the viral transcriptional machinery might activate telomere resolution are discussed.

B. Materials and Methods

Cells and Viruses. BGMK cells (African Green Monkey) obtained from ATCC were grown in monolayer culture in Dulbeco modified eagles medium (DME, Gibco Laboratories) supplemented with 10% newborn calf serum (Gibco Laboratories). Human thymidine kinase-minus (TK$^-$) H143 cells used to select recombinant vaccinia viruses were obtained from D. Panicali and grown in monolayer culture in DME supplemented with 5% fetal calf serum and 20 µg/ml 5-bromo-2'-deoxyuridine (Sigma).

Vaccinia virus (strain WR) obtained from ATCC was propagated by the infection of suspension cultures of HeLa S3 cells which were a generous gift from V. Paetkau. Shope fibroma virus (Kasza strain) was propagated by the infection of monolayer cultures of BGMK cells. Virus stocks were prepared from cells harvested by centrifugation at 48 hours post-infection and then swelling and douncing the cell pellet (Condit & Motyczka, 1981, Wills *et al.*, 1983). Virus titres were determined by infecting monolayers of 1.6×10^6 BGMK cells with 10 fold serial dilutions of the crude virus stocks. After one hour adsorption in a 200 µl volume, fresh medium was added and the infections were allowed to proceed for 48 hours at which time the cells were fixed, stained with 0.1% crystal violet and foci were counted.

The recombinant vaccinia virus vCST-2a was generated by the standard mechanism of homologous recombination between a plasmid containing a fusion of the SFV TRT and a bacterial chloramphenicol acetyl transferase (CAT) reporter cassette flanked by vaccinia virus thymidine kinase (TK) gene sequence into the vaccinia virus (TK) gene (Mackett *et al.*, 1984). The insertion vector used in this study, pVV5.1, has been previously described (Franke *et al.*, 1985). This vector was cleaved at unique sites with restriction enzymes *Sal*I and *Bam*HI, and the linearized vector, with the vaccinia virus 7.5 kilodalton gene (7.5k) promoter removed, was purified and blunt-ended with T4 DNA polymerase in the presence of equal concentrations of the four deoxyribonucleotides. This vector was used as the recipient for a DNA fragment which had been excised from pCST-1 (described in the following section) with *Bam*HI and *Sst*I and the overhanging ends blunted with T4 DNA polymerase. The resulting insertion vector pVCST-1 consisted of the CAT gene under the regulation of the minimal TRT sequence of SFV, flanked on both sides by vaccinia virus TK sequences. Recombinant TK$^-$ viruses were selected on H143 cells in the presence of 5-bromo-2'-deoxyuridine and screened for the presence of the insert by dot blot hybridization with a CAT gene specific probe. The insertion site of the TRT-CAT sequence was confirmed by Southern blotting.

Plasmids and Strains. All bacterial plasmids which contained inverted repeats of viral sequence were maintained in E. coli strain DB1256 (DeLange *et al.*, 1986) and all other plasmids were propagated in E. coli strain HB101. The construction of all plasmids which contain inverted repeats of wild-type SFV sequence and the deletion derivatives of this sequence have been described previously (DeLange *et al.*, 1986, DeLange & McFadden, 1987). Plasmids which contained large inverted repeats of SFV telomere sequence pSAB-67, pSXB-102, pSCB1a were all derived by removing the appropriate *Acc*I, *Xho*I or *Cla*I fragment from the plasmid pYSF1-30 described previously (DeLange *et al.*, 1986) and subcloning these fragments into the *Sma*I site of pUC 19. The plasmid pSCX-1 was derived from pSCB1a by cleavage with *Xho*I to delete the central axis 0.7 kilobase (kb) fragment and self-ligation of the vector to generate a palindromic insert with a central axis *Xho*I site (Fig. III-6a).

Plasmids which contain the SFV TRT sequences upstream of a promoterless CAT gene were generated by excising one half of the inverted repeat of pSD-19 (DeLange *et al.*, 1986) or deletion derivatives of this construct as a *Hind*III/ *Afl*II fragment. The overhanging 5'-ends of these fragments were filled with T4 DNA polymerase and the blunted fragments were ligated into the *Sma*I site of the vector pMTL-24 (Chambers *et al.*, 1988). The telomere deletions of interest were then isolated from the pMTL constructs as

*Kpn*I fragments and cloned into the *Kpn*I site of pUC19-CAT 4, which contains the CAT gene cloned into the *Sma*I site of pUC 19 oriented such that CAT can be placed under the control of viral sequences introduced into the *Kpn*I, *Sst* I or *Eco*RI sites (Macaulay & McFadden, 1989). The prototype member of this series of vectors, pCST-1 contains the entire wild-type TRT sequence of SFV obtained from the vector pSAD-2 (DeLange & McFadden, 1987) directing expression of the CAT gene.

Point mutations were generated in the TRT by blunt-end ligating an *Eco*RI/ *Afl*II fragment from pSAD-2 into the *Sma*I site of M13mp19. This ligation destroys the *Eco*RI site but regenerates the central axis *Afl*II site. Oligonucleotide directed site specific mutagenesis was performed (Kunkel *et al.*, 1987) and the appropriate mutations were identified by dot blot hybridization and then confirmed by sequencing. The mutated *Eco*RI / *Afl*II fragments were isolated and self-ligated or ligated to a fragment of wild-type sequence to generate inverted repeats which were subcloned into the *Sst* I site of pUC 19 and used for assays of telomere resolution. For promoter assay the mutated fragments were ligated into the vector pUC19-CAT-4 so that the sequences could be tested for their ability to direct CAT expression. All of the plasmid DNAs that were used in transfection assays to measure resolution or promoter activity were purified by a single round of isopycnic centrifugation in CsCl.

Analysis of Telomere Resolution in Transfected Cells. The ability of the wild-type or mutated TRT inverted repeat sequences to be resolved into linear minichromosomes with hairpin termini was assayed as previously described (DeLange *et al.*, 1986). Plasmid DNA containing inverted repeat telomere sequences was transfected into previously infected monolayers of $1.6x10^6$ BGMK cells and total DNA was harvested 24 hours after transfection. This DNA was treated with restriction enzyme *Dpn*I to cleave the input unreplicated DNA, electrophoresed through 0.7% agarose gels, transferred to nitrocellulose paper, hybridized with nick translated plasmid DNA probes and visualized by autoradiography.

Promoter and Transcript Analysis. BGMK cells which had been infected with virus and transfected with plasmid DNA were harvested 20 hours post-infection and the lysates used for CAT assays as modified for SFV-infected cells (Macaulay & McFadden, 1989). Semiconfluent monolayers of BGMK cells in 3.5 cm wells of 6-well dishes were adsorbed with SFV (M. O. I= 1) for one hour in a 200 µl volume. Each well was then overlayed with 2.5 mls of DME and then 200 µl of calcium-phosphate precipitate containing 500 ng of plasmid DNA was added to each well. The calcium-phosphate precipitate was made by first adding the purified plasmid DNA to 100 µl of filter sterilized 2 x NNH (0.818 g NaCl, 0.6 g Hepes [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 20.1 mg $Na_2HPO_4.7H_20$; pH 7.1, in a 50 ml volume), followed by adding 100 µl of filter sterilized, freshly prepared 250 mM $CaCl_2$. This solution was rapidly mixed by bubbling filtered air through the solution. This solution was then allowed to precipitate for 30 min prior to its application to the infected cell monolayer. Three hours after the application of the precipitated plasmid DNA each monolayer was washed with warm phosphate buffered saline (PBS; 145 mM NaCl, 4 mM KCl, 10 mM phosphate pH 7.2) plus 5 mM ethylenediamine tetraacetic acid (EDTA), and then with PBS and 3 mls of fresh DME was added to the cell monolayers. The cultures were harvested 20-24 hours post-infection by scraping the infected cells into 1 ml of PBS, pelleting in a 1.5 ml microfuge tube, and then resuspending the cells in 100µl of 250 mM Tris-HCl (pH 8.0). The cells were freeze-thawed three times by alternating between a dry ice ethanol bath and a 37° heat block, and vortexing vigorously each time the cells were thawed. The cell debris was pelleted (12000 x g 1 min.) and the CAT activity in the lysate was assayed. A 20 µl aliquot of the cell lysate was mixed with 70 µl of 250 mM Tris-HCl pH 8.0, 35 µl H_2O, 1 µl ^{14}C-chloramphenicol (0.1 mCi/ml, 54.2 mCi/mmole, New England Nuclear research products), and 20 µl of freshly made 4 mM acetyl CoA (Pharmacia). This was incubated at 37°C for 10-20 min. and then the sample was extracted with 1 ml of ethyl acetate. The ethyl acetate was placed in a fresh 1.5 ml microfuge tube and the solvent was evaporated in a speed vac concentrator (Savant). The residue was resuspended in 20 µl of ethyl acetate and the acetylated forms of

^{14}C-chloramphenicol were separated by thin layer chromatography on silica gel coated (0.2 mm) plastic sheets (Merck). The entire sample was spotted onto the plates and they were developed in a chamber saturated with 95% chloroform, 5% methanol. The plates were air dried, lightly coated with En^{3}hance (Dupont) and exposed to X-ray film (X-OMAT AR, Kodak) In each experiment an equal amount of purified plasmid DNA was transfected into the poxvirus infected cells and equal amounts of each lysate were used in each assay for CAT activity. No attempt was made to accurately quantitate the CAT activity, only a positive or negative result was recorded.

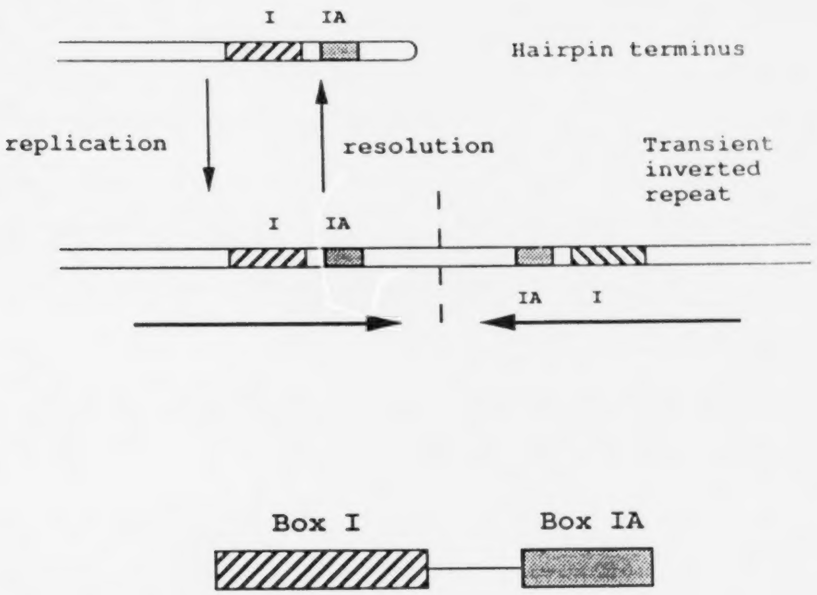
RNA synthesis driven from the TRT promoter in vaccinia virus recombinants was analyzed by primer extension as follows. Monolayers of BGMK cells in 100 mm dishes were infected with the recombinant vaccinia virus vCST-2a at a multiplicity of 10 pfu/ cell; at 2 and 16 hours post infection RNA was harvested from the infected monolayers by scraping the cells into guanidinium isothiocyanate (Sigma) and pelleting the RNA through cesium chloride (Chirgwin *et al.*, 1979). 40 μg of this RNA was hybridized with 200 ng of a 5'-end-labelled 17 mer oligonucleotide primer which was complementary to the CAT gene. This primer was extended with avian myeloblastosis virus reverse transcriptase (Life Sciences Inc.) in the presence or absence of dideoxynucleotides as described previously (Bertholet *et al.*, 1987). The extended products of these reactions were electrophoresed through 8% polyacrylamide sequencing gels containing 8 M urea and visualized via autoradiography.

Unless specified all of the restriction and modification enzymes used in this study were obtained from Bethesda Research Laboratories and used under the conditions recommended by the manufacturer.

C. Results

Similarity between consensus TRT and late promoter sequences.

During the productive replication cycle of poxviruses, the hairpin termini are transiently


I
IA
Hairpin terminus
replication
resolution
Transient inverted repeat
I
IA
IA
I
Box I
Box IA

SFV	TTTTTTTCTAG GGTTA TAAATTA
VAC	TTTTTTTCTAG ACAC- TAAATAA
CPV	TTTTTTTCTAG ACAC- TAAATAA
RCN	TTTTTTTCTAG ACAT- TAAATAA

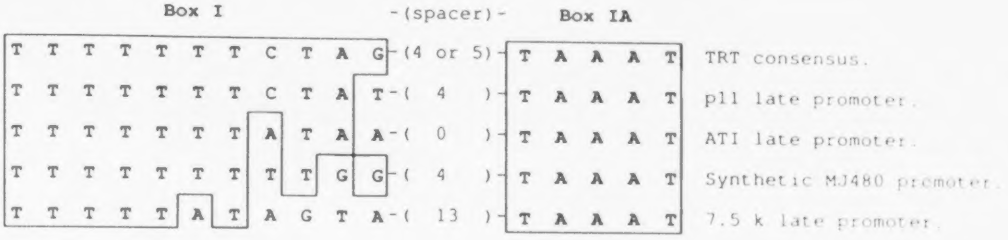
Figure III-1. The upper panel illustrates the poxviral telomere in the mature hairpin form and in the inverted repeat configuration which exists transiently during DNA replication. The inverted repeat structure is resolved by *trans*-acting viral factors to yield two daughter hairpin telomere structures. The relative positions of boxes I and IA of the minimal TRT sequences are indicated, but extrahelical bases on the hairpin and non-palindromic sites on the inverted repeat are not shown. The lower panel indicates one copy of the TRT sequence of Shope fibroma virus (SFV), vaccinia virus (VAC), cowpox virus (CPV) and racoonpox virus (RCN).

converted into the inverted repeat replicative intermediate which is the substrate for *trans*-acting viral factors that catalyze the isomerization of these sequences into two daughter hairpin termini (DeLange & McFadden, 1990). As shown in Fig. III-1, the minimal sequence domain required for resolution consists of two blocks of sequence, designated as box I and IA, that are highly conserved among poxviruses and which are separated by a spacer of 4 or 5 nucleotides that has diverged. Inspection of the conserved sequences which comprise box I and IA reveal a strong similarity with the consensus structure of vaccinia late promoters (Davison & Moss, 1989). As can be seen in Fig. III-2, the p11 late promoter is different by only one nucleotide from the consensus TRT within the aligned box I and IA domains, and many of the other characterized late promoters have a conserved TAAAT motif which corresponds exactly to the TRT box IA.

Transcription directed by the TRT sequences of SFV. To investigate the possibility that the the TRT sequence of SFV is capable of directing orientation specific transcription we constructed a series of plasmid vectors in which the bacterial CAT gene was placed under the regulation of well characterized poxviral promoters or a single copy of the TRT sequence and a series of mutated versions of the TRT that had been created for resolution assays. CAT activity assayed from cells harvested at late times showed that the TRT was capable of directing gene expression in an orientation specific manner (Fig. III-3 lanes 5 and 6). The effectiveness of the TRT to function as a promoter can be inferred by comparing the amount of CAT activity directed from the TRT with the levels from the SFV T1 early promoter (Fig. III-3, lane 2) and the vaccinia virus 7.5 k promoter (Fig. III-3, lane 1).

A series of deletions of the SFV telomere sequence indicate that the minimal TRT sequence in boxes I and IA are the essential sequence elements for promoter activity. $5'$-deletions which remove nucleotides from the sequence TTTTTTT of box I (Fig. III-4 lane 7) or $3'$-deletions into the TAAAT of box IA (lane 8) eliminate the promoter activity of the

Telomere Resolution Target = Late Promoter?



Box I											-(spacer)-	Box IA					
T	T	T	T	T	T	T	C	T	A	G	-(4 or 5)-	T	A	A	A	T	TRT consensus.
T	T	T	T	T	T	T	C	T	A	T	-(4)-	T	A	A	A	T	p11 late promoter.
T	T	T	T	T	T	T	A	T	A	A	-(0)-	T	A	A	A	T	ATI late promoter.
T	T	T	T	T	T	T	T	T	G	G	-(4)-	T	A	A	A	T	Synthetic MJ480 promoter.
T	T	T	T	T	A	T	A	G	T	A	-(13)-	T	A	A	A	T	7.5 k late promoter.

Figure III-2. The TRT is very similar to poxvirus late promoters. The TRT sequence which is conserved among all of the poxviruses for which the sequence is available (TRT consensus) is shown aligned with the sequences of; the vaccinia virus 11 kDa gene late promoter (p11 late promoter), cowpox A type inclusion body promoter (pATI late promoter), an artificial strong late promoter synthesized to match the determined consensus sequence for vaccinia virus late promoters (Davison & Moss, 1989) (Synthetic MJ480 promoter), and the vaccinia virus 7.5 kDa gene late promoter (7.5 K late promoter).

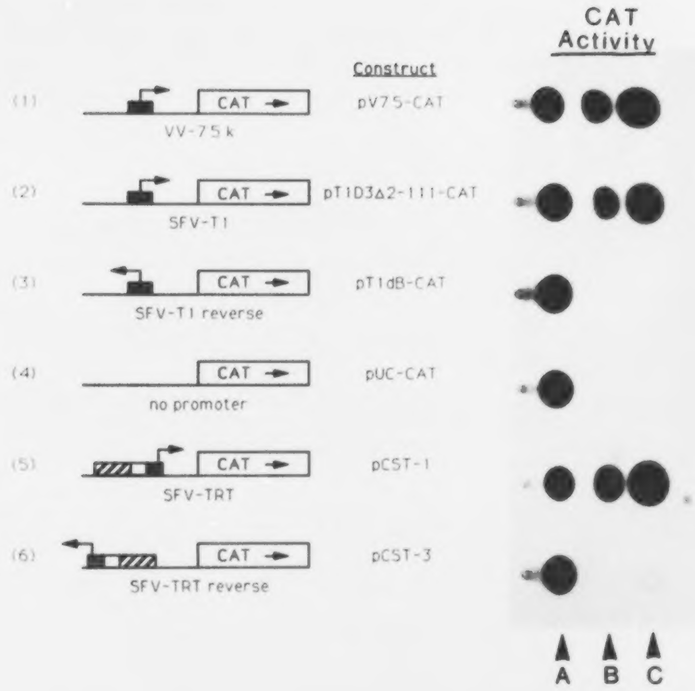

CAT
Activity
Construct
(1)
CAT
VV-7.5 k
pV7.5-CAT
(2)
CAT
SFV-T1
pT1D3Δ2-111-CAT
(3)
CAT
SFV-T1 reverse
pT1dB-CAT
(4)
CAT
no promoter
pUC-CAT
(5)
CAT
SFV-TRT
pCST-1
(6)
CAT
SFV-TRT reverse
pCST-3
A
B
C

Figure III-3. Promoter properties of the SFV TRT sequence. Plasmid constructs containing the CAT gene downstream of the vaccinia virus 7.5 kDa early/late promoter (pV7.5-CAT), the two orientations of the Shope fibroma virus T1 early promoter (pT1D3'Δ2-111-CAT), (pT1dB-CAT), no promoter (pUC-CAT), or the two orientations of the Shope fibroma virus TRT (pCST-1), (pCST-3) were tested for their ability to express CAT activity in transient assay when transfected into SFV-infected cells. The shadings of box I and IA are the same as in Fig. III-1. A, B, and C at the bottom of the figure indicate chloramphenicol, 1-acetate chloramphenicol, and 3-acetate chloramphenicol respectively.

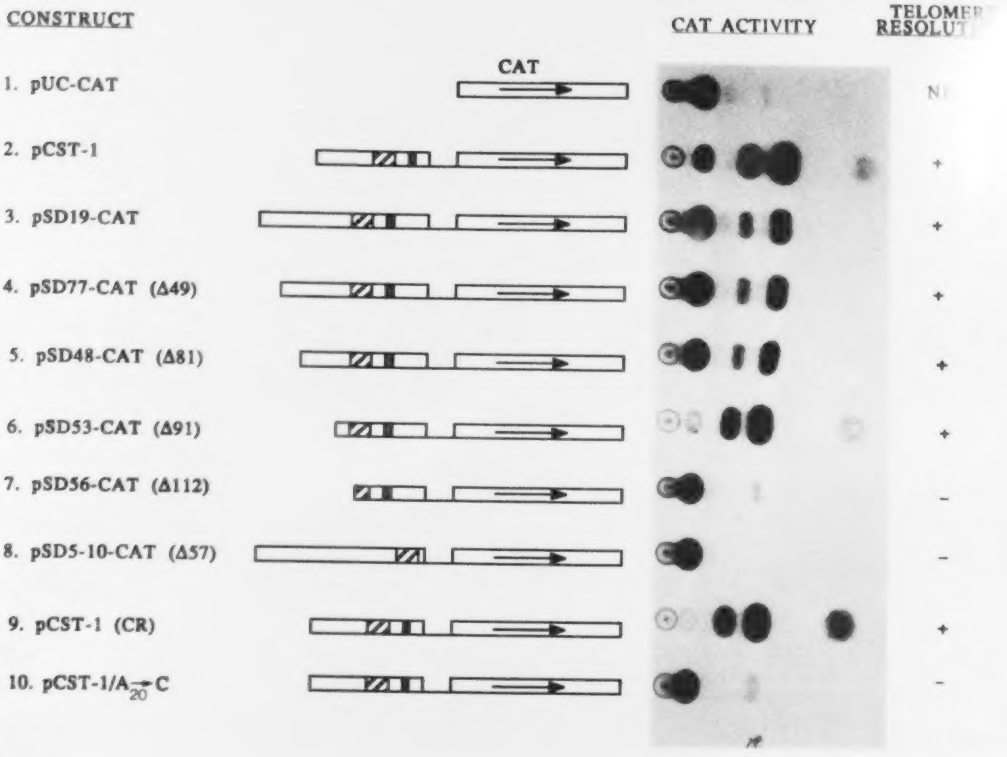

CONSTRUCT
CAT ACTIVITY
TELOME
RESOLU
CAT
1. pUC-CAT
2. pCST-1
3. pSD19-CAT
4. pSD77-CAT (Δ49)
5. pSD48-CAT (Δ81)
6. pSD53-CAT (Δ91)
7. pSD56-CAT (Δ112)
8. pSD5-10-CAT (Δ57)
9. pCST-1 (CR)
10. pCST-1/A$_{20}$→C
+
+
+
+
+
-
-
+
-

Figure III-4. CAT assay demonstrating the minimal TRT sequences responsible for promoter activity. CAT assays of infected/transfected cells were performed as for Fig. III-3. pSD19-CAT (lane 3) contains all of the sequences in pCST-1 (lane 2) but also includes 46 basepairs of 3'sequence which extends to the axis of symmetry of the replicative intermediate inverted repeat. This 3'sequence contains several start and stop codons which result in a decreased expression of CAT activity in relation to pCST-1. The constructs in lanes 4 through 7 are 5'deletions of the parent pSD19-CAT. pSD5-10-CAT (lane 8) is a 3'-deletion of pSD19-CAT which completely removes the TRT box IA. The number of nucleotides deleted in each case is indicated in brackets. The cloning of pCST-1 resulted in the deletion of nucleotide A$_{16}$ which was restored by site specific mutagenesis to wild-type in pCST-1 (CR) (lane 9). In pCST-1/A$_{20}$→C the TAAAT sequence of box IA was mutated to TAACT. In the the telomere resolution column (+) indicates that the plasmid containing this inverted repeat was resolved *in vivo* into monomer and multimer linear minichromosomes. The (-) symbol indicates that the plasmid was replicated but not resolved. The resolution data shown in lanes 1 through 9 have been presented previously (DeLange *et al.*, 1986, DeLange & McFadden, 1987), and resolution data for pCST-1/A$_{20}$-C are shown in Fig. III-7c.

TRT. It has been observed that mutations within the conserved TAAAT sequence of vaccinia virus promoters abrogate promoter function (Davison & Moss, 1989). When the sequence TAAAT in box IA of the TRT is mutated to TAACT the ability of the TRT to direct gene expression is lost (Fig. III-4 lane 10). The mutated versions of the TRT have been tested in an *in vivo* resolution assay for their ability to support the resolution of inverted repeats into hairpin structures (DeLange *et al.*, 1986, DeLange & McFadden 1987). Deletions from the 5'-side which remove sequence from box I or from the 3'-side which remove box IA of the TRT on one or both sides of the inverted repeat fail to support *in vivo* hairpin resolution. In fact the same deletion constructs that define the 5'-and 3'-boundaries of the TRT in resolution assays also define the domain of the promoter function of the SFV TRT. The same result has been observed for point mutations which alter the highly conserved TAAAT of box IA in terms of promoter activity (Fig. III-4, lane 10) and resolution (Fig. III-7, last section). Thus a clear correlation can be demonstrated between the ability of the TRT to direct transcription and the ability to support the resolution of inverted repeats *in vivo*.

SFV TRT functions as a late promoter in a transient expression assay. Previously it has been shown that an early SFV promoter, T1, will function correctly in a transfection assay in cells infected with SFV or vaccinia virus (Macaulay & McFadden, 1989). CAT assays on lysates from infected/transfected cells indicate that the TRT sequence of SFV can also direct the expression of CAT activity in cells which had been infected with either SFV or vaccinia virus (Fig. III-5, lanes 1 and 2). Thus the TRT is recognized as a promoter when fused as a single copy to the CAT gene and as a resolution signal when in an inverted repeat configuration by both Leporipoxviruses and Orthopoxviruses. When the DNA replication inhibitor cytosine arabinofuranoside (ara C) is used to treat cells during poxvirus infection, late gene expression and DNA replication are prevented (Moss, 1990). In the presence of ara C SFV-infected cells continue to direct the expression of CAT activity from the SFV T1 early promoter; however, very little CAT

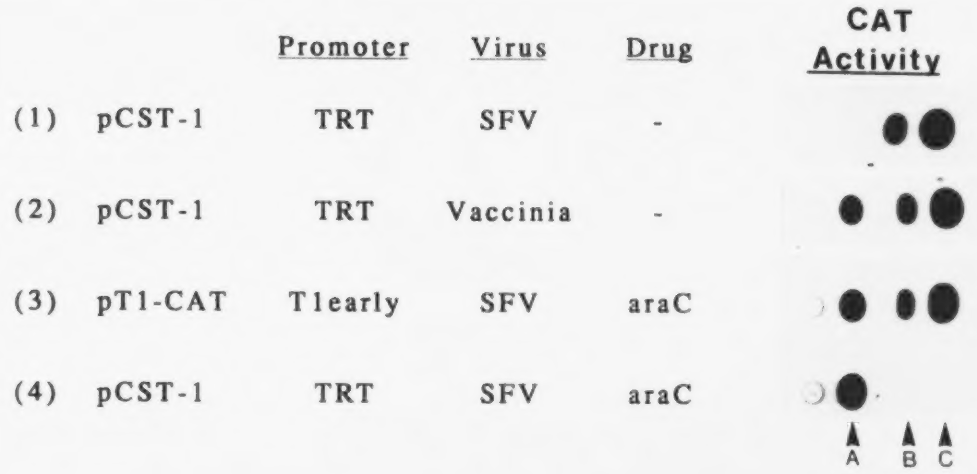


Figure III-5. The TRT sequence is functional as a promoter only at late times during infection. Lanes 1 and 2 illustrate the promoter function of the SFV TRT when it is transfected into cells infected with either SFV or vaccinia virus. A transfected CAT gene under the regulation of an early promoter (SFV T1) is expressed when viral DNA replication and late gene expression are blocked with ara C. A, B, and C at the bottom of the figure refer to chloramphenicol, 1-acetate chloramphenicol, and 3-acetate chloramphenicol respectively.

activity is observed to be directed from the TRT (Fig. III-5, lanes 3 and 4) suggesting that the TRT can function as a promoter primarily at late times during infection.

SFV TRT functions as a late promoter in a recombinant vaccinia virus. The ability of vaccinia virus *trans*-acting factors to recognize and utilize the TRT of SFV as a late promoter prompted us to test the ability of the TRT to act as a promoter from within the context of the viral genome. A recombinant vaccinia virus, vCST-2a, was generated in which the TRT-directed CAT gene was inserted into the vaccinia TK gene (Fig. III-6). The 5'-start site of RNA collected at 16 hours post-infection was mapped by primer extension using a CAT specific oligonucleotide probe. The extended products from this primer display a number of characteristics that are common to vaccinia virus late transcripts (Fig. III-6b). As can be seen from the population of sizes whose diversity appears to begin within the TAAAT motif of Box IA (lanes 1-4), the 5'-end is heterogeneous in length and is likely to be polyadenylated in a fashion similar to late orthopoxvirus genes (Bertholet *et al.*, 1987, Schwer *et al.*, 1987). The length of the apparent 5'-poly (A) head of the TRT-CAT RNA is about 20 nucleotides, again consistent with other late poxviral transcripts. The terminal 5'-sequence of the TRT-driven RNA which is complementary to the DNA sequence is within the TAAAT corresponding to the TRT box IA. Thus, in this context within the TK gene, the SFV TRT behaves as a rather typical late promoter.

Another late promoter sequence can function to resolve replicative intermediates. The observation that the minimal TRT sequence can act as a late promoter led us to investigate the possibility that other late promoters could, if presented in an inverted repeat configuration, function as a viral telomere to be resolved into hairpins. To test this, we first constructed a series of large inverted repeats of SFV telomere sequence (Fig. III-7a).Plasmids pSAB-67, pSXB-102 and pSCB-1a all contain the native TRT within progressively larger inverted repeats and can be efficiently resolved into linear minichromosomes *in vivo* (Fig. III-7b, lanes 1, 2 and 3). However, in the construct shown in lane 4 the central axis *Xho*I fragment of pSCB-1a was removed to generate pSCX-1,

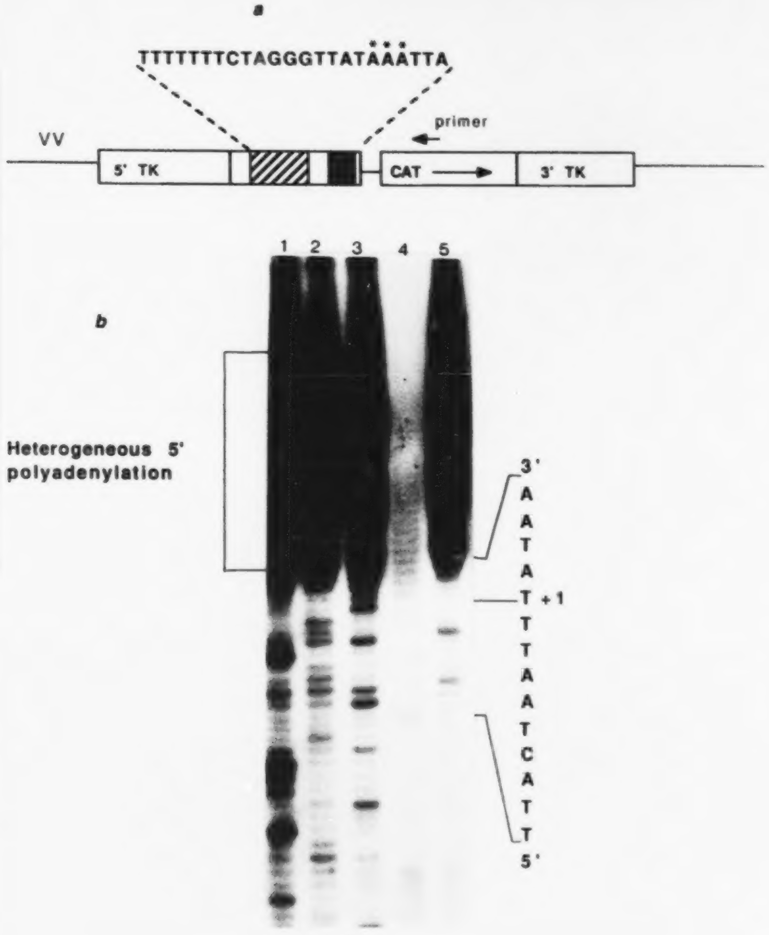

a
TTTTTTTCTAGGGTTATAAATTA
primer
VV
5' TK
CAT
3' TK
1 2 3 4 5
b
Heterogeneous 5' polyadenylation
3'
A
A
T
A
T +1
T
T
A
A
T
C
A
T
T
5'

Figure III-6. A recombinant vaccinia virus (VV) containing the TRT sequence of SFV directs the expression of a CAT reporter gene. (a) The plasmid pVCST-1 was used to construct the recombinant vaccinia virus VCST-2a with the TRT regulated CAT gene inserted into the vaccinia virus TK gene. The relative position and orientation of the primer used for the RNA analysis are indicated and the apparent RNA start sites are marked with asterisks. (b) Primer extension analysis of the TRT specific RNAs produced at late times during infection with vCST-2a. End-labelled primers were extended in the presence of deoxynucleotides and dideoxynucleotides: ddCTP (lane 1), ddATP (lane 2), ddTTP (lane 3), ddGTP (lane 4) or in the absence of dideoxynucleotides (lane 5). The nucleotide sequence of the DNA template is shown where +1 indicates the first nucleotide that is complementary to the RNA. Lane 4 was underloaded but the sequence of interest is still visible.

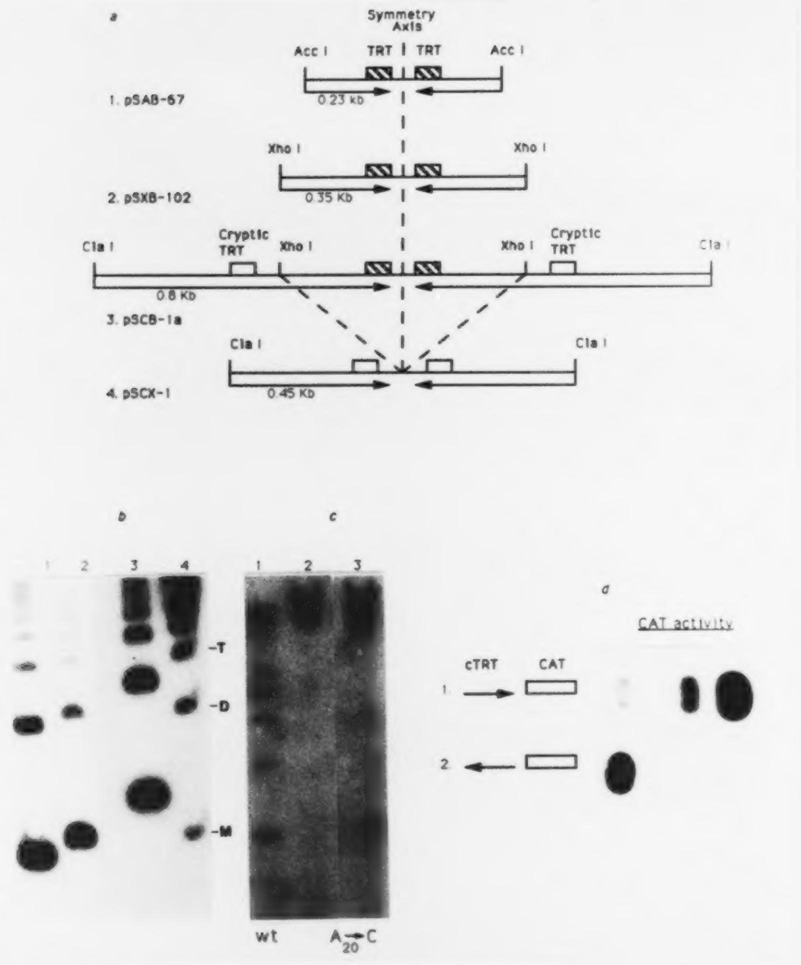

a
Symmetry Axis
Acc I
TRT
TRT
Acc I
1. pSAB-67
0.23 kb
Xho I
Xho I
2. pSXB-102
0.35 Kb
Cla I
Cryptic TRT
Xho I
Xho I
Cryptic TRT
Cla I
0.8 Kb
3. pSCB-1a
Cla I
Cla I
4. pSCX-1
0.45 Kb
b
1
2
3
4
-T
-D
-M
c
1
2
3
wt
A20→C
d
CAT activity
cTRT
CAT
1
2

Figure III-7. Identification of a cryptic TRT (cTRT) sequence in SFV that is capable of acting as a late promoter and a resolution target. (a) Plasmids in which the SFV termini have been cloned in an inverted repeat configuration to mimic the replicative form of the virus. pSAB-67, pSXB-102 and pSCB-1a consist of various lengths of viral telomere sequence, and all include the conserved TRT near the axis of symmetry of the inverted repeat. pSCX-1 was generated by the removal of a 0.7 kb *Xho*I fragment from the central axis of plasmid pSCB-1a. Ligation of the resulting *Cla*I- *Xho*I fragments produced a 0.9 kb perfect palindrome of viral DNA with a new central axis *Xho*I site and lacking the native TRT. (b) Southern blot of *Dpn*I resistant DNA harvested from SFV-infected cells which were transfected with pSAB-67 (lane 1), pSXB-102 (lane-2), pSCB-1a (lane 3) or pSCX-1 (lane 4). The blot was hybridized with nick translated pUC 19 [^{32}P] DNA so that the monomer and multimer forms of only the plasmid DNA constructs are visualized. (c) Southern blot illustrating the lethal effect of a point mutation within the critical TRT box IA sequence. DNA was harvested from infected cells which had been transfected with pSD-19,(wild-type TRT [wt]; lane 1), pUC-19, (no TRT) (lane 2), or pCST-1/$A_{20}\rightarrow C$.(lane 3) (d) CAT assay demonstrating the ability of a single copy of the cTRT to act as a promoter. Lane 1 shows the *Cla*I/ *Xho*I fragment of pSCX-1 fused to CAT such that the *Cla*I site forms the 5'-end of the insert. Lane 2, same vector with the pSCX-1 fragment in the opposite orientation.

which contains a 0.9 kb perfect palindrome of viral DNA that lacks the native central axis including the entire TRT sequences. Despite the removal of the SFV TRT domain pSCX-1 can be replicated and resolved *in vivo* into monomer and multimer linear minichromosomes (Fig. III-7b, lane 4). This result is particularly striking when one considers that all of the palindromes of non-viral origin which have been tested in the resolution assay have always been inactive (McFadden *et al.*, 1988). As a comparison, the ability of this "cryptic" TRT to catalyze resolution to minichromosomes is compared to the complete ablation of resolution by a single point mutation in the TRT box IA (Fig. III-7c, lane 3) which had been shown previously to destroy promoter activity of the TRT (Fig. III-3, lane 10). Since the remaining SFV sequence in pSCX-1 had not been assayed for promoter activity we fused one half of the pSCX-1 inverted repeat to a CAT reporter gene in both orientations as was done previously with the native SFV TRT and used this as a vector for transient assay in SFV-infected cells. The pSCX-1 fragment was also found to be capable of directing CAT expression in an orientation specific manner (Fig. III-7d). Examination of the SFV sequence in pSCX-1 yielded no regions which exactly fit the TRT consensus sequence, but 5'-deletion analysis identified the sequence TACGTTTACACCTATATAAAT as a strong late promoter element in the transient expression assay (data not shown). The 3'-T of the TAAAT of this sequence maps 96 nucleotides upstream of the *Xho*I site which forms the symmetry axis of pSCX-1, and so the position and orientation of this "cryptic" TRT is very similar to that of the native TRT in the viral replicative intermediate. Notice that since the two strands at the symmetry axis of the resolution target segregate into the daughter hairpins (DeLange & McFadden, 1990, Merchlinsky, 1990) the minichromosome generated from pSCX-1 will represent viral telomeres with the *Xho*I sequences now at the hairpin turn around. Furthermore, since pSCX-1 is a perfect palindrome, there can be no "flip" and "flop" isomers and all of the termini of pSCX-1 minichromosomes would be identical in sequence.

The relative positions of the TRT and the cTRT in the mature and replicative intermediate

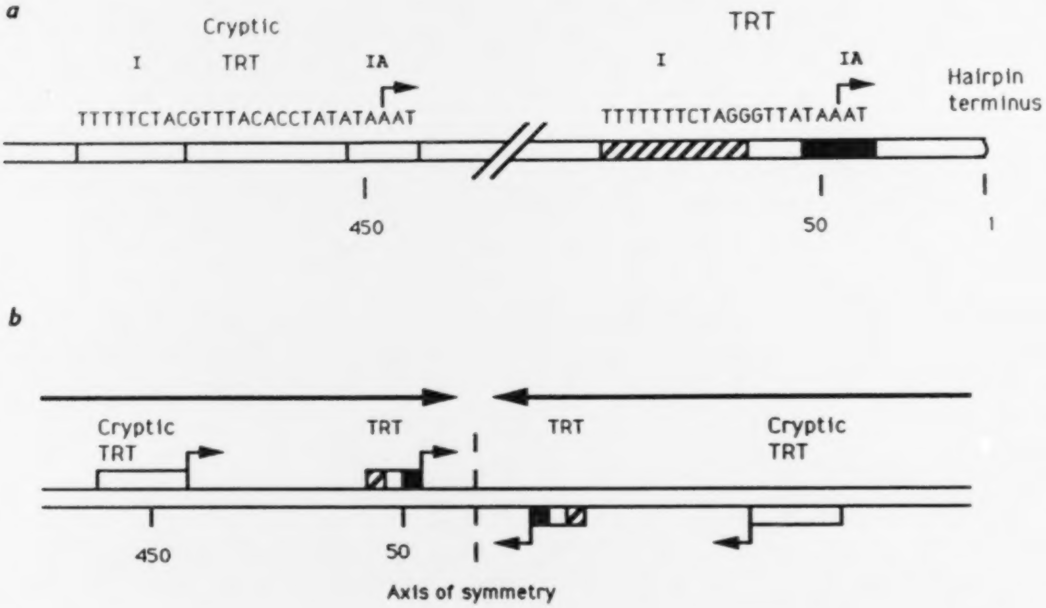

a
Cryptic
TRT
I
IA
TTTTTCTACGTTTACACCTATATAAAT
450
TRT
I
IA
TTTTTTTCTAGGGTTATAAAT
50
Hairpin
terminus
1
b
Cryptic
TRT
TRT
TRT
Cryptic
TRT
450
50
Axis of symmetry

Figure III-8. Organization of the TRT and cTRT within the telomere of the SFV in the mature hairpin form as packaged in the virion (a) and in the inverted repeat replicative intermediate configuration (b). The arrows indicate the RNA start sites which have been determined by primer extension (TRT) from constructs containing fusions to the reporter CAT gene. Whether these RNAs are synthesized from their telomeric location in SFV during resolution *in vivo* remains to be established.

form of the SFV telomere are illustrated in Fig. III-8. To date, we have been unable to observe RNA transcripts initiating at the TRT or the cTRT sites when these sequences are at their normal telomeric location in SFV. It remains to be determined whether transcription and resolution are interdependent, or possibly mutually exclusive activities of the same sequence.

D. Discussion

We have undertaken an investigation of the relationship between viral transcription, which synthesizes RNA, and the resolution of poxvirus replicative intermediates, which is a site-specific DNA recombination event. The involvement of transcriptional events in the process of DNA replication is well known from work on the mammalian papovaviruses such as SV40 and polyoma where transcriptional elements are known to be required for the initiation of DNA replication (DePamphilis, 1988, Hassell *et al.*, 1986). Similarly transcription has been shown to "silence" replication origins (Patel & Bastia, 1986) and can act as a terminator or block to replication forks (Brewer & Fangman 1988). To aid our investigation we have taken advantage of the previous observation that cloned inverted repeat versions of poxvirus telomeres are replicated and resolved into linear hairpin molecules when transfected into virus-infected cells (DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). Similarly, the regulation of viral promoters can be studied by transient assays in infected cells and in recombinant poxviruses. Both transcription and resolution are unique to infected cells and it is believed that virus encoded factors are responsible for both activities.

A significant relationship between late transcription and telomere resolution can be drawn from a number of observations. In this work we show that the TRT sequence responsible for telomere resolution in SFV is also capable of acting as a promoter at late times during infection. This capacity to drive transcription has been observed both in transient assay and when the sequence is integrated within the TK gene of a recombinant viral genome. All mutated versions of the TRT which have thus far been investigated (point mutations, 3'- and 5'-deletions) show a direct correlation between the ability of a single copy of the TRT to function as a promoter and the ability of the inverted repeat configuration to support telomere resolution into hairpin termini. Domain analysis indicates that the conserved regions of the known TRT sequences from SFV and several Orthopoxviruses (Fig. III-1b) are very similar to the consensus sequence of viral late promoters (Fig. III-2). The overall variation in the catalogue of sequences of characterized late promoters may indicate the need for the genes which they regulate to be expressed at different levels (Davison & Moss, 1989). On the other hand the apparent sequence conservation of the TRT domains may be a reflection of the requirement for a specific degree of promoter activity to obtain an optimal amount of telomere resolution at the proper time during the viral replicative cycle. This argument is supported by the observation that another late promoter sequence, denoted "cryptic" TRT (cTRT), that maps over 0.4 kb from the native viral terminus will also function to resolve inverted repeats. The cTRT is less than 50% identical to the native TRT in terms of primary sequence and yet supports telomere resolution at an appreciable efficiency, whereas all other control palindromes tested are uniformly negative. Thus telomere resolution might be at least partly regulated by the affinity of the TRT sequence for transcription factors which are available at late times during infection. The similarity of the TRT sequence to late promoters thus suggests a novel mechanism for limiting DNA processing events to a particular temporal stage of the viral life cycle.

The terminal regions of the vaccinia virus DNA genome contain a large region of non-coding repeats. Nevertheless, vaccinia transcripts oriented toward the terminus have been detected in this region (Parsons & Pickup, 1990). The SFV termini contains a series of 9 tightly packed open reading frames all of which are expressed at early times with transcription being directed toward the telomeres (Macaulay *et al.*, 1987). The T1 open reading frame is the most proximal gene to the SFV terminus and three signals to terminate early transcription have been identified between T1 and the hairpin (Upton, *et al.*, 1987). This clustering of termination signals may be a fortuitous observation or it could be interpreted as an indicator of the importance of preventing early transcription from entering the non-coding region of the termini.

Transcription of the viral telomeres alone is clearly not sufficient for the resolution of inverted repeats. The structural requirements that the replicative intermediates be nearly perfect palindromes with the TRT sequences in a particular orientation and within a specific distance of one another have been previously demonstrated (McFadden *et al.*, 1988, Merchlinsky, 1990). This is further illustrated by the observation that the cTRT identified in SFV is a homologue of a vaccinia sequence that directs transcription into the telomere at late times (Parsons & Pickup, 1990). However, the SFV cTRT apparently has no effect upon telomere resolution until two opposing copies are moved within 100 bp of one another in an inverted repeat configuration. Genetic studies of vaccinia virus which have attempted to determine the specific alleles important for telomere resolution have identified only mutants which are defective in some aspect of late gene expression (DeLange, 1989, Merchlinsky & Moss, 1989b) This has been interpreted as an indication that some late gene product(s) are essential for telomere resolution (Merchlinsky & Moss, 1989b). Most of these mutants show defects in the RNA polymerase and it could also be argued that the lack of telomere resolution results from the defect in either late RNA synthesis or even simply promoter recognition by elements of the late transcription apparatus. One of the mutants which is defective in telomere resolution but competent for late transcription, *ts* 22, has been characterized (DeLange, 1989, Merchlinsky & Moss 1989b, Pacha & Condit, 1985). This mutation shows a resolution negative phenotype and is defective for the production of late gene products due to the degradation of the viral mRNA. Another mutant, *ts* 9383, is defective for resolution and has been mapped to one of the subunits of the capping enzyme (DeLange, 1989).

While transcription of the telomeres at late times appears to be linked in some fashion to the resolution of replicative intermediates, the question of the precise role played by RNA synthesis remains open. It has been shown that transcription *in vivo* can transiently alter the local topology of the template DNA (Giaever & Wang, 1988, Liu & Wang, 1987, Tsao *et al.*, 1989). Specifically, it has been demonstrated that the movement of an RNA polymerase complex along duplex DNA can induce two supercoiled domains of opposite sign which balance one another. The generation of positive superhelical density which occurs ahead of a transcription complex is presumably relaxed by the activity of a topoisomerase. Indeed, it has recently been shown that in eukaryotic cells topoisomerase I may operate in close conjunction with RNA polymerase II and is localized to actively transcribed genes (Stewart *et al.*, 1990). However an absolute requirement for the relaxation of tension accumulated in transcribed DNA has yet to be established and topoisomerase inhibition studies suggest that such an absolute need may not exist (Schaak *et al.*, 1990). It is known that transcription with its associated superhelical tension can proceed through palindromes without causing the extrusion of a cruciform or other major topological alterations (Morales *et al.*, 1990). However, the effect of creating transcripts from promoters which are arranged in a head to head fashion, such as exist at poxvirus telomeres, has not been addressed.

The detailed significance of convergent promoters to telomere resolution remains to be worked out. One model which can be envisaged involves the formation of open complexes by an RNA polymerase complex simultaneously on either side of an inverted repeat. This could result in sufficient unwinding of the DNA helix to allow the formation of a secondary structure such as a Holliday junction analog which could be cleaved by recombination activities encoded by poxviruses (Evans *et al.*, 1988). A model for cruciform extrusion has been proposed to include site specific unwinding by a TRT-specific helicase activity (McFadden *et al.*, 1988). The present data suggest an alternative activity, namely promoter dependent unwinding, which could accomplish the same goal. Confirmation of the mechanism for resolution will require an *in vitro* resolution system, which this work indicates must include at least some elements of the late transcriptional machinery.

The utilization of promoter sequences to resolve replicative intermediates is an elegant example of how viruses have evolved to utilize a pre-existing element for multiple functions. In some eukaryotes it has been shown that actively transcribed DNA is preferentially involved in homologous recombination (Thomas & Rothstein, 1989). Similarly a strong correlation has been demonstrated between active transcription and site-specific recombination of the immunoglobulin kappa genes (Blackwell *et al.*, 1986, Schlissel & Baltimore, 1989). However, we believe that this is the first example of transcriptional events implicated in activating a site-specific recombination event in eukaryotic viruses.

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Chapter IV

Dual Usage of Viral Late Promoters in Transcription and Resolution of Poxvirus Replicative Intermediates

A. Introduction

Poxviruses like vaccinia virus and Shope fibroma virus (SFV) have double stranded linear DNA genomes with terminal inverted repeats (TIRs) of 10 kb for vaccinia (Garon *et al.*, 1978) and 12.4 kb for SFV (DeLange *et al.*, 1984). The extreme terminal region of poxvirus genomes consists of covalently closed incompletely basepaired hairpin-loops that can exist in two isomeric forms; "flip" and "flop" due to the presence of unpaired bases in the hairpin loop (Baroudy *et al.*, 1982, DeLange *et al.*, 1986). The significance of the unpaired bases is unknown; however, the conservation of this characteristic among poxviruses suggests that they may serve an important function. The termini of vaccinia virus possess a series of short, noncoding, tandemly repeated sequences (Wittek & Moss 1981), the function of which is not clear. The number of the repeated sequences and their arrangement is variable among strains of vaccinia virus due to homologous recombination among and between the repeats (Baroudy *et al.*, 1982). The termini of SFV have no tandemly repeated noncoding sequences. Rather the TIRs of SFV consist primarily of sequence encoding a tightly packed series of tandemly arranged genes. All of the genes in the TIRs of SFV are expressed at early times during infection and are oriented such that they are transcribed toward the telomere (Upton *et al.*, 1987, Macaulay, *et al.*, 1987).

The replicative cycle of poxviruses occurs exclusively within the cytoplasm of the host cell with little or no involvement of the nuclear replicative or transcriptional apparatus (Prescott *et al.*, 1971, Hruby *et al.*, 1979). Replication of poxvirus DNA produces high molecular weight concatemers of nascent viral genomes fused end-to-end in a linear array. In this configuration the TIR sequence transiently exists as a large imperfect inverted repeat that forms a junction between adjacent genomes (Moyer & Graves, 1981). Maturation and packaging of the nascent poxvirus genomes requires that the concatemeric replicative intermediate be cleaved and processed to form linear monomer genomes with mature hairpin termini (reviewed by DeLange & McFadden, 1990). The resolution of poxvirus replicative intermediates is dependent upon the presence of a twenty basepair *cis*-acting segment of DNA that is located near the hairpin termini. The *cis*-acting telomere resolution target (TRT) (DeLange & McFadden, 1990, Merchlinsky & Moss, 1989b), is highly conserved among poxviruses. In order to function in the resolution of telomere replicative intermediates, the TRT sequence must be present in two copies arranged in an inverted orientation with respect to one another (Merchlinsky, 1990). Cloned versions of SFV or vaccinia virus telomere replicative intermediates can be resolved into hairpins when transfected into cells infected with either SFV or vaccinia virus (DeLange *et al.*, 1986). This suggests that the TRT is part of a conserved mechanism used by poxviruses to resolve their replicative intermediates and to maintain the integrity of their hairpin termini.

The sequence of the TRT bears a striking similarity to the recently determined consensus for viral late promoters (Davison & Moss, 1989) and in fact corresponds to one of the strongest naturally occurring viral promoters. A plasmid borne TRT sequence is capable of acting as a strong viral late promoter in transient assays. The TRT sequence also acts as a promoter when it is incorporated in a heterologous position into the genome of a recombinant vaccinia virus vector (Stuart *et al.*, 1991). It has also been determined that the TRT sequence of vaccinia virus directs RNA synthesis from its native telomeric location at late times during infection (Hu & Pickup, 1991). Several different sized transcripts can be detected when vaccinia virus late RNA is probed with sequences from the extreme genomic termini (Parsons & Pickup, 1990). Typical of poxvirus late transcripts, the RNA initiated from the TRT appears as a heterogeneous smear and carries a variable length polyadenylate stretch at its 5' end (Stuart *et al.*, 1991). Other RNA species that are detected with probes against the extreme terminal sequence of vaccinia virus originate from a sequence that lies 1.4 kb upstream from the TRT (Parsons & Pickup, 1990). SFV encodes a homolog of this promoter, that we have designated cryptic TRT (cTRT) (Stuart *et al.*, 1991). The cTRT is conserved in sequence and genome organization between vaccinia and SFV. The function of these telomeric promoters is unclear. The TRT and cTRT are the only terminal sequences that are well conserved between vaccinia virus and SFV. In the case of SFV, the TRT and cTRT are the only late promoters thus far identified in the terminal region, yet they do not direct the expression of any open reading frames. In each case no more than five amino acids could be translated before a stop codon is reached. The transcription initiated from each of these telomeric promoter sequences at late times can be directed through the inverted repeat telomere replicative intermediate and into an adjacent viral genome.

It has been proposed that transcription from the TRT is required for the resolution of telomere replicative intermediates. This could occur by several possible mechanisms: by making the sequence accessible to recombination factors, by the synthesis of a catalytic RNA species that takes part in the reaction, by causing unwinding of the DNA helix near the termini and inducing the formation of a secondary structure that acts as a target for "resolution factors", or by aiding in the binding of "resolution " factors (Stuart *et al.*, 1991). We have investigated the role of transcription and promoter elements in the resolution of telomere replicative intermediates by taking advantage of the observation that cloned versions of poxvirus replicative intermediates are replicated and resolved when transfected into poxvirus infected cells (DeLange *et al.*, 1986). Results from experiments in which RNA synthesis was inhibited at various times throughout the course of the infection suggest that the resolution of cloned poxvirus replicative intermediates *in vivo* can not be unlinked from the process of active transcription. Other experiments in which the native TRT sequences were replaced by well characterized promoters of viral or bacterial origin show that some but not all viral promoters can facilitate telomere resolution if placed in the correct context . These data suggest that the ability of the TRT sequence to act as a viral promoter is necessary but not sufficient for the resolution of telomere replicative intermediates. The transcriptional apparatus, but not the act of transcriptional elongation alone is an essential component of the viral resolution machinery.

B. Materials and Methods

Cells and viruses. BGMK cells (African green monkey) and RK13 cells (rabbit kidney) were obtained from the ATCC. These cell lines were maintained as monolayer cultures in Dulbeco's modified Eagle's medium (DME), supplemented with 10% heat inactivated newborn calf serum (Gibco laboratories). HeLa S3 cells (a gift from Dr. V. Paetkau) were grown as suspension cultures in Joklik modified Eagle's medium (Flow laboratories) supplemented with 10% heat inactivated equine serum (Gibco laboratories). SFV (Kasza strain) and vaccinia virus (WR) were obtained from the ATCC. The thermolabile vaccinia virus mutant ts53 which is defective in the expression of late genes at the non-permissive temperature was a gift from Dr. R. Condit. Vaccinia virus recombinant VTF7-3 which expresses the phage T7 RNA polymerase (Fuerst *et al.*, 1986) was a gift from Dr. B. Moss. Crude stocks of the wild type and recombinant vaccinia viruses were prepared by infection of HeLa S3 cells at a multiplicity of 1 plaque forming unit (pfu) per cell. Following a 48 hr incubation period virus was harvested by centrifugation and Dounce homogenization. Virus was then partially purified by sedimentation through sucrose as described (Joklik, 1962). Stocks of the temperature sensitive vaccinia virus were prepared by low multiplicity infection of BGMK cells at 31°C (Condit & Motyczka, 1981). SFV stocks were prepared by the infection of monolayers of 3 x 10^9 BGMK cells at a multiplicity of one focus forming unit (ffu) per cell. The virus was harvested and prepared as described (Wills *et al.*, 1983).

Plasmids and Strains. All of the plasmids that contain a replicative intermediate version of the SFV or vaccinia virus telomeres (pSAD-2, pSD19, pSD56, pSAlB56a, pVCB5a) were previously described (DeLange *et al.*, 1986, DeLange & McFadden, 1987) All plasmids that contain viral promoters in an inverted repeat configuration were constructed as follows (also see Fig. IV-3); a plasmid carrying the promoter of interest was linearized at a site 3' to the promoter region. The linearized plasmid was purified from low melting point agarose (Langridge *et al.*, 1980) or was isolated from a polyacrylamide gel by soaking the gel slice in 10 mM Tris-HCl (pH 8.0) for 16 hrs and then extracting the purified DNA with phenol:chloroform (1:1) and chloroform. The purified DNA was then self-ligated at high concentration to obtain concatemers. The ligated DNA was then cleaved 5' to the promoter region and the DNA was fractionated on an 8% native polyacrylamide gel. The inverted repeat fragment corresponding to a dimer of the promoter sequence was excised as a gel slice, crushed and eluted in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA overnight at 37°C with shaking. The DNA was purified away from acrylamide by filtration through a 0.2 µm filter then concentrated by multiple extractions with 2-N-butanol. Following extraction with an equal volume of chloroform the dimer fragment was dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA and ligated into the appropriate site in the vector pUC19. The cowpoxvirus A type inclusion body promoter was obtained from the plasmid p1246 (Patel *et al.*, 1988) as a *Cla*I-*Bgl* II fragment. The vaccinia virus 11 kDa and 7.5 kDa gene promoters were obtained from the plasmids pSC20 (Buller *et al.*, 1988) and pVV 5.1 (Franke *et al.*, 1985) as *Bgl*II-*Xho*I and *Sal*I-*Bam*HI fragments respectively. The promoter for the SFV T1 open reading frame was obtained from pT1a (Macaulay & McFadden, 1989) as a *Hind*III-*Eco*RI fragment. The phage T7 promoter was taken from pGEM-2 as a *Hind*III-*Pvu*II fragment. All plasmids that carried inverted repeat sequences were cloned and propagated in the rec$^-$ Escherichia coli (E. coli) strain DB1256 (DeLange *et al.*, 1986), all other plasmids were maintained in HB101 or JM83.

Initially, we attempted to construct an inverted repeat with the vaccinia virus p11 promoter sequence, but we were unable to recover any correct clones. We found that some part of the 100 bp sequence 5' to the vaccinia 11 kDa protein gene acted as a promoter in E. coli. This was determined by transforming E. coli JM83 with the plasmid pSC20 and selecting the transformants with ampicillin. All of the ampicillin resistant colonies turned blue when plated on agar containing 4-chloro-5-bromo-3-indolyl-B-D-galactoside (X-Gal) indicating that the LacZ gene was being expressed. A derivative of pSC20 from which the p11 promoter had been removed produced only white colonies when transformed into JM83. The ability to function as a promoter appears to render an inverted repeat fusion of the p11 sequence highly recombinogenic in E. coli. In order to overcome this problem oligonucleotide directed site specific mutagenesis (Kunkel *et al.*, 1987) was used to convert a single copy of the TRT sequence into the vaccinia virus 11 kDa gene promoter sequence. This construct could be stably propagated in an E. coli strain bearing multiple rec^- lesions.

RNA preparation and analysis. RNA was isolated from 100 mm dishes of BGMK cells that had been infected with SFV at a multiplicity of 5 ffu per cell for 17 hours (late RNA) or for 17 hours in the presence of 100 µg/ml cycloheximide (early RNA). Inhibitors of protein synthesis prevent the uncoating of the infecting poxvirus cores. This treatment enhances the synthesis of early RNA by preventing the repression of early gene expression that occurs following the initiation of viral DNA replication (Woodson, 1967, McAuslan, 1963). The infected cells were harvested by scraping up the monolayers in RNA lysis buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate pH 7.0, 0.5% sodium N-laurylsarcosine and 0.1M β-mercaptoethanol). The total RNA was isolated by an acid-phenol partitioning procedure (Chomczynski & Sacchi, 1987) and then poly A^+ RNA was selected by affinity chromatography using oligo d(T) cellulose (Aviv & Leder 1972). The poly A^+ RNA was fractionated by electrophoresis through 1.5 % agarose gels containing 2.2 M formaldehyde (Sambrook *et al.*, 1989). RNA was transferred to a Hybond C transfer membrane in 10 X SSC, U.V. cross-linked to the membrane and then probed with [^{32}P] labelled random primed DNA probes (Feinberg & Fogelstein, 1984) or strand specific DNA probes generated as described (Hu & Messing, 1986) and visualized by autoradiography.

The activity of the TRT promoter in the presence or absence of the RNA chain terminator cordycepin was determined as follows. 1 x 10^6 BGMK cells in 35 mm dishes were infected with 2 pfu per cell of the recombinant vaccinia virus VCST-2a which expresses the bacterial chloramphenicol acetyl transferase (CAT) gene under the regulation of the SFV TRT promoter sequence (Stuart *et al.*, 1991). The virus was applied in 200 µl of pre-warmed DME plus 10% new born calf serum. After a 30 min adsorption period at 37°C, 2.5 mls of fresh medium were added to each dish and the infection was allowed to proceed for 5 hrs, at which time the medium was aspirated from half of the monolayers and replaced with fresh medium containing 20 µg of cordycepin per ml. Following a 1 hr incubation all of the monolayers were washed with warm phosphate buffered saline (PBS; 145 mM NaCl, 4 mM KCl, 10 mM phosphate pH 7.3) plus 5 mM EDTA, and then with PBS. The monolayers were then overlayed with 1 ml of warm serum free DME that was supplemented with 30 µCi of [^{3}H]-uridine. Monolayers of infected cells that had been pre-treated with cordycepin were also pulse labelled in the presence of 20 µg per ml cordycepin. The infected cells were pulse labelled for 30 min after which the medium was removed and the monolayers were washed twice with cold PBS and then harvested by the addition of 400 µl of RNA lysis buffer to each monolayer. Total RNA was extracted from the infected cell homogenates by the acid phenol procedure (Chomczynski & Sacchi, 1987), and DNA was removed by incubation at 37°C for 20 min in the presence of RNAse free DNase. One half of each RNA preparation was heated to 65°C for 5 min and then hybridized to filters on which 20 µg of the plasmid pCST-1 (which contains the CAT gene) had been denatured and immobilized. Following hybridization and stringent washing the filters were counted to determine the quantity of newly synthesized [^{3}H] labelled CAT RNA. The other half of each sample was was spotted on to Whatman GFC filters and precipitated with ice cold 10% trichloroacetic acid (TCA), 20 mM sodium pyrophosphate (Na PPi). After three washes in 10% TCA, 20 mM Na PPi the acid insoluble counts on each filter were determined. Each labelling condition was performed in triplicate and an average value is presented.

In vivo assay for replicative intermediate resolution. The resolution of poxvirus replicative intermediates was assayed as described previously (DeLange *et al.*, 1986). Briefly, monolayers of 1-2 x10^6 RK13 or SIRC cells were infected with SFV (Kasza strain) at a multiplicity of 1 ffu per cell. The virus was applied in a 200 µl volume and was gently agitated to allow the virus to adsorb to the cells for 1 hr at 37°C, after which the monolayers were overlayed with 2.5 mls of fresh medium. At this time the infected cells were transfected by the addition of 50 ng of $CaPO_4$ precipitated plasmid DNA. After a 3 hr incubation, unadsorbed DNA and virus were removed by washing the cells with PBS plus 5 mM EDTA and then with PBS. Each of the infected/transfected monolayers was then overlayed with fresh medium. After a 24 hr incubation the total cell DNA was collected by lysing the cells on the plates by the addition of 10 mM Tris-HCl, 10 mM EDTA, 0.5% SDS, and 2 mg/ml proteinase K. The cell lysates were incubated at 37°C for 4 hours and then nucleic acids were purified by sequential extraction with equal volumes of phenol, phenol:chloroform (1:1) and chloroform. Following precipitation and washing with ethanol each DNA sample was resuspended in 50 µl of 10 mM Tris-HCl, 1 mM EDTA and treated with 50 µg/ml RNase A. Total cellular DNA was digested with restriction enzyme *Dpn*I which cleaves in methylated input plasmid DNA but not DNA that has been replicated in mammalian cells, and 500 ng of each sample were loaded into each lane of a 0.7% agarose gel. DNA species were fractionated by electrophoresis, transferred to a nitrocellulose membrane, and fixed to the membrane by baking at 80°C for two hrs. Replicated plasmid species were then detected by probing the filter with randomly primed [^{32}P] labelled plasmid vector sequences. Finally the blots were visualized by autoradiography.

To determine the effect of inhibition of RNA synthesis on the resolution of poxvirus replicative intermediates, an *in vivo* transfection assay was performed as described above except that at 4, 6, 8, 10, and 12 hrs after transfection one infected/transfected monolayer was harvested to determine the normal extent of resolution. At each time point a duplicate culture was washed with PBS and had its medium replaced with fresh medium containing 20 µg of cordycepin per ml of medium and maintained until the 18 hr time point at which time all of the remaining cultures were harvested and the DNA was analyzed by southern blot hybridization as described above.

Enzymes and chemicals. All of the nucleic acid restriction and modification enzymes that were used in this study were obtained from Bethesda Research Laboratories and were used as directed by the manufacturer. 3' deoxyadenosine (cordycepin) was obtained from the Sigma Chemical Co. and was made up as a 200 µg per ml stock in either DME or PBS and stored for no more than 2 days at 4°C. [^{32}P] ATP and [^{3}H]-uridine were purchased from ICN Radiochemicals.

C. Results

The extreme terminal non-coding regions of the Orthopoxvirus vaccinia virus are transcribed at late times during infection. The terminal sequences of vaccinia virus and the Leporipoxvirus SFV have very little homology; however, the two viruses share the presence of late promoter elements within their hairpin termini. While the viral DNA is being replicated, the terminal region transiently exists in an inverted repeat configuration schematically depicted in figure IV-1A. Transcription of the SFV inverted repeat replicative intermediate is directed at least in part by the TRT promoter sequence (Stuart *et al.*, 1991). However, the transcripts generated from the TRT in its telomeric location have been difficult to map precisely due to the potential for the 5' ends of the transcripts to anneal into an intramolecular RNA hairpin loop. A single arm of the inverted repeat replicative intermediate was used to create a single stranded probe for northern analysis of SFV late RNA . The transcripts identified by the SFV telomere probe are heterogeneous in length as judged by the smear that is visualized when late RNA is annealed with a single stranded DNA probe designed to detect transcripts that are directed toward the termini (Fig. IV-1 B). No discrete RNA species could be identified; however, the greatest intensity of signal appears to be centered just below the 18 S marker. Thus, the majority of the transcripts correspond roughly in size to twice the length of the SFV non-coding terminal region suggesting that the transcripts may pass through the axis of symmetry of the inverted repeat and proceed into an adjoining genome. While this is the most interesting possibility, another explanation is that the telomeric transcripts are generated by the viral transcription

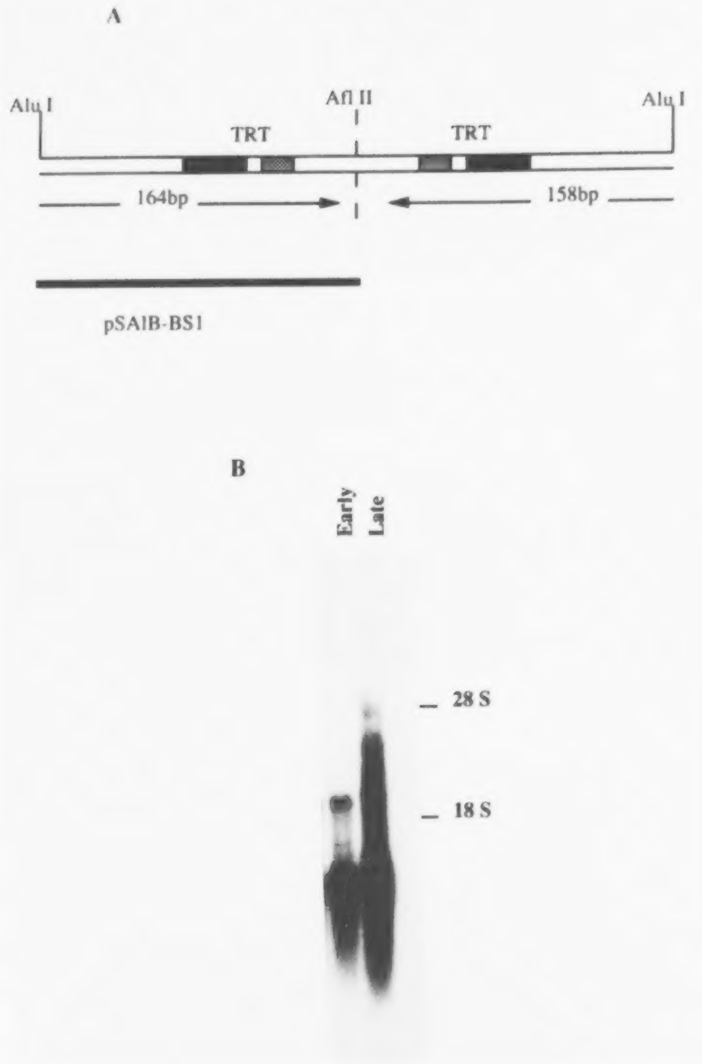

Figure IV-1.(A) Schematic representation of the inverted repeat replicative intermediate of SFV. The axis of symmetry that forms the hairpin terminus in the mature viral genome is indicated by the dashed line and the recognition sequence for *Afl*II. The relative positions of the TRT sequences required for replicative intermediate resolution are indicated and the conserved box I and box Ia motifs are indicated by the filled boxes. The thick black line indicates the 164 bp telomere fragment that was used as a probe for a Northern blot of SFV RNA. (B) A Northern blot of SFV RNA probed with the pSAIB-BS1 fragment. Early and late RNA were harvested at 17 hrs in the presence of 100 µg/ml cycloheximide (Early) or at 17 hrs without treatment (late). The position to which the cellular 28 S and 18 S cellular ribosomal RNAs migrated are indicated to the right of the figure as a size standard.

complex progressing around the hairpin termini of mature genomes. The only way to distinguish between these two possibilities would be to sequence the RNA or cDNAs made from the telomeric transcripts.

The possibility that active transcription of the poxvirus replicative intermediates played a role in telomere resolution encouraged us in an effort to investigate the connection between the processes of transcription and telomere resolution. The poxvirus RNA polymerase is resistant to the effects of α-amanitin and no other specific inhibitor drugs are available. We chose to inhibit transcription *in vivo* with the RNA precursor analog 3' deoxyadenosine (cordycepin). This compound acts as an RNA chain terminator and we hoped that it would specifically inhibit transcription without having pleiotropic effects upon nucleic acid metabolism. Cordycepin should be a particularly effective inhibitor of poxvirus late transcription because viral late RNA is initiated with a variable length pseudo-templated polyadenylate stretch (Bertholet *et al.*, 1987). In a preliminary experiment we tested the efficacy of a spectrum of cordycepin concentrations on the inhibition of incorporation of [^{3}H]-uridine into viral late RNA initiated from the TRT promoter (Table IV-1). Based upon these results it was determined that the inclusion of 20 μg of cordycepin per ml of culture medium could completely inhibit the incorporation of [^{3}H]-uridine into nascent viral RNA sequences.

We then utilized this information to assay the effect of inhibiting RNA synthesis upon the resolution of cloned poxvirus replicative intermediates *in vivo*. Cell monolayers were infected with SFV and transfected with a cloned viral replicative intermediate (pSAD-2). Over a 16 hr period the untreated cultures displayed a progressive increase in the amount of plasmid DNA that was resolved into linear minichromosomes. When cordycepin was added to the infected/transfected cultures at 4 hrs or at 6 hrs post-transfection an inhibition of minichromosome formation was observed (data not shown). Since the inhibition of transcription prevented the resolution of the pre-existing inverted repeat telomere fusions, we extended our investigation to a mutational analysis of the TRT promoter sequence.

	Cordycepin concentration ug/ml					
	0	0.01	0.1	1.0	10.0	20.0
total TCA ppt. counts	11222 ±1551	12068 ±994	10701 ±1099	9796 ±878	1278 ±136	148 ±18
Counts hybridizing to CAT	115 ±5	116 ±8	123 ±6	79 ±7	5 ±9	2 ±9

Table IV-1. The effect of cordycepin concentration on the incorporation of $[^3H]$-uridine into newly synthesized viral RNA. Samples of pulse labelled RNA isolated at 5 hrs post-infection from cells infected with the recombinant vaccinia virus VCST-2a which expresses the CAT gene under the regulation of the SFV TRT sequence, were divided in half and one aliquot was precipitated with TCA to determine the total precipitated counts. The remainder of the sample was hybridized to a CAT specific probe and the counts that were incorporated into CAT RNA were determined. Each number is given as an average of three samples ± the standard deviation.

Mutations that are lethal to the ability of the TRT sequence to act as a promoter are also lethal for the resolution of replicative intermediates. The substitution of a C for T within the highly conserved ATTTA motif is lethal to telomere resolution when the substitution occurs symmetrically on both sides of the inverted repeat (Fig. IV-2A, lane G, and IV-2B lane G/G). However, the same mutation is not lethal to resolution if it is made on only one side of the inverted repeat (Fig. IV-2A, lanes wt/G and G/wt, Fig. IV-2B wt/G and G/wt). A cloned inverted repeat version of the SFV TRT sequences with this particular point mutation in only one copy of the TRT sequence can be resolved albeit with a considerable decrease in efficiency. The T to C substitution made within box Ia has been shown to abrogate viral transcription directed by the TRT sequence (Stuart *et al.*, 1991) and the same mutation made within the vaccinia virus 11 kDa gene late promoter has also been shown to abrogate transcription (Davison & Moss, 1989). We therefore investigated the possibility that promoter function alone might be enough to confer resolution on an cloned replicative intermediate.

Fusions of viral and nonviral promoters were constructed and used as target molecules in an *in vivo* transfection assay. The procedure used to construct the inverted repeat substrate plasmids is displayed in Fig. IV-3. The inverted repeat plasmid generated by the fusion of promoter sequences of interest were tested for *in vivo* resolution by transfecting the plasmids into poxvirus-infected cells. Resolution was scored by the appearance of a ladder of *Dpn*I resistant monomer and multimer linear hairpin terminated minichromosomes. Inverted repeat fusions of poxvirus early promoters (SFV T1 promoter and vaccinia virus 7.5 kDa gene early promoter) were not resolved at early or at late times after transfection (Fig. IV-4B, lanes 2 and 3). These plasmids were also not resolved when transfected into cells that had been infected with the thermolabile vaccinia virus mutant ts53 at the nonpermissive temperature. Vaccinia virus ts53 is able to synthesize early gene products and to replicate its DNA at the nonpermissive temperature but is unable to initiate transcription from late promoters (Hooda-Dhingra *et al.*, 1989). Some of the late promoter

A

	TRT sequences							Resolved in vivo
	I		Ia		Ia		I	
wt	TTTTTTTTCTAG	GGTTA	TAAAT	----	ATTTA	TAACC	CTAGAAAAAAAA	++
G	TTTTTTTTCTAG	GGTTA	TAAgT	----	AcTTA	TAACC	CTAGAAAAAAAA	-
wt/G	TTTTTTTTCTAG	GGTTA	TAAAT	----	AcTTA	TAACC	CTAGAAAAAAAA	+
G/wt	TTTTTTTTCTAG	GGTTA	TAAgT	----	ATTTA	TAACC	CTAGAAAAAAAA	+

B

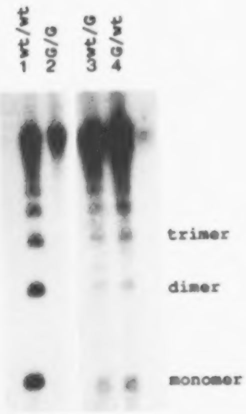

Figure IV-2. (A) Resolution of cloned SFV replicative intermediates and site specific mutants of the replicative intermediate. The wild type TRT sequence is indicated as wt, the variant "G" contains a symmetric site specific A-to-G substitution within box Ia, wild type sequences are in capitals and mutations are indicated in lower case letters. wt/G and G/wt each have a single nucleotide substitution in one arm of the inverted repeat and wild type sequence in the other arm. The ability of these sequences to function as resolution substrates *in vivo* is indicated to the right of each sequence. Wild type resolution efficiency is indicated by ++, no resolution by - and some resolution but less than wild type by +. (B) A Southern blot of the plasmid DNA indicated in panel A. Each plasmid was transfected into SFV infected cells and the harvested *Dpn*I resistant DNA was probed with [^{32}P] labelled pUC-19. The size of the replicated plasmid species is indicated as monomer, dimer or trimer of the original plasmid DNA.

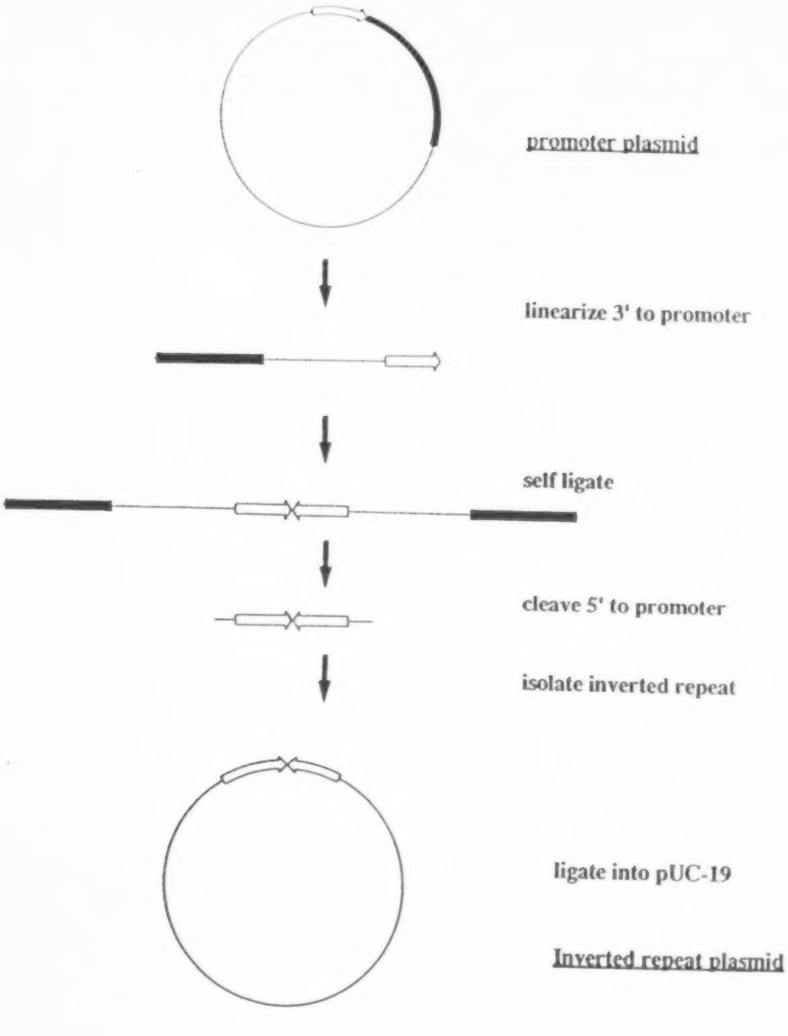
promoter plasmid
linearize 3' to promoter
self ligate
cleave 5' to promoter
isolate inverted repeat
ligate into pUC-19
Inverted repeat plasmid

Figure IV-3. The construction of plasmids carrying inverted repeat configurations of promoter sequences. The promoter of interest is cleaved at its 3' end, then self ligated and cleaved at its 5' end. Dimer size fragments can then be isolated by polyacrylamide gel electrophoresis and these can be ligated into a pUC vector and propagated in a rec$^-$ E. coli strain. Inverted repeat plasmids were used to test the ability of a variety of promoter sequences to function as resolution substrates *in vivo*.

sequences that we tested in this assay could function in *cis* as targets for the resolution of inverted repeat structures. The ability of the TRT and cTRT to function as resolution sequences has been shown previously (Stuart *et. al.*, 1991). The vaccinia virus 11 kDa gene promoter is a strong late promoter that is identical to the TRT within the two conserved regions of sequence (box 1 and box 1a) that are critical for both telomere resolution and efficient late transcription. Not surprisingly this sequence can function as a TRT when cloned in an inverted repeat configuration and transfected into poxvirus infected cells (Fig. IV-4B, lane 4). Vaccinia virus and SFV are capable of utilizing the cowpoxvirus A type inclusion body promoter (ATI) at late times during infection (Patel *et al.*, 1988). The ATI sequence functions as a strong late promoter and has a conserved ATTTA motif but is not utilized as a TRT for the resolution of inverted repeats by SFV (Fig. IV-4B, lanes 7). The original construction of the ATI inverted repeat placed the two ATTTA motifs in direct opposition to one another with no nucleotide pairs separating them (pATId). A 10 bp oligonucleotide linker identical to the sequence of the SFV hairpin loop was inserted to separate the ATTTA sequences (pATId-AL); however, this did not confer the ability of the construct to be resolved in poxvirus infected cells (Fig. IV-4B, lane 8). The use of a heterologous RNA polymerase along with a cognate recognition sequence for the polymerase allowed a test of the effect of transcription on an inverted repeat. A bacteriophage T7 RNA polymerase recognition sequence was obtained from the plasmid vector pGEM-2 and cloned in an inverted repeat configuration (pGT7d) such that the promoters direct the synthesis of RNA toward one-another (Fig. IV-5A). This construct was replicated but failed to be resolved when transfected into cells that had been infected with wild type vaccinia virus (WR strain) or with the recombinant vaccinia virus VTF7-3 (Fig. IV-5B, lane 1). This was in contrast to the replication and resolution of pVCB5a which contains a cloned version of the vaccinia virus telomere replicative intermediate (Fig. IV-5B, lanes 2 and 3). The recombinant vaccinia virus used in this experiment expresses the phage T7 RNA polymerase at both early and late times during infection. The ability of

A

	Viral Promoters	Resolved in vivo
SFV T1	CGTTAATTTCTTAAAAAAAAGTCAATTATA$\dot{A}$GTAA	NO
VV 7.5 kDa	TAAAAGTAGAAAATATATTCTAATTTATT$\dot{G}$CACG	NO
TRT	GCTAGGTTTATACTTTTTTTTCTAGGGTTA$\underline{T\dot{A}\dot{A}\dot{A}T}$	YES
cTRT	CCCCGTGTTTTTCTACGTTTACTCCTATA$\underline{T\dot{A}\dot{A}\dot{A}T}$	YES
VV 11kDa	AGAATTTCATTTTGTTTTTTTCTATGCTA$\underline{T\dot{A}\dot{A}\dot{A}T}$	YES
CPV ATI	GTTAAGTTTTGAATAAAATTTTTTTATAA$\underline{T\dot{A}\dot{A}\dot{A}T}$	NO

B

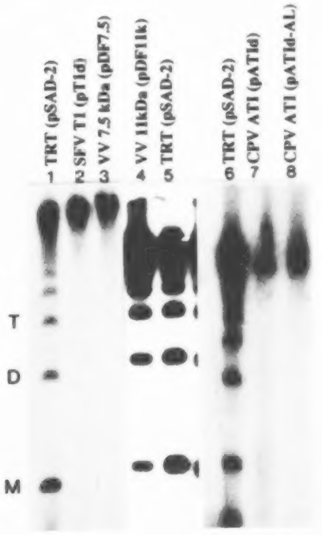

Figure IV-4. The ability of different viral promoters to function as resolution substrates when cloned in an inverted repeat configuration. (A) The poxviral promoter sequences are indicated and the transcriptional start sites are indicated with asterisks. Each of these promoters was cloned in an inverted repeat configuration such that transcription was initiated in a divergent fashion. The ability of the plasmids carrying these dimer inserts, to be resolved is indicated with a simple yes or no. (B) Southern blot analysis of the total DNA harvested from SFV infected cells that were transfected with the various plasmids carrying inverted repeats of promoter sequences. The name of the promoter is indicated above each lane and the name of the plasmid that contains an inverted repeat of the promoter sequence is bracketed. Resolution is scored positive by the appearance of a ladder of *Dpn*I resistant linear minichromosomes, see for example lane 1 (pSAD-2). Monomer (M), dimer (D), and trimer (T), linear minichromosomes are indicated.

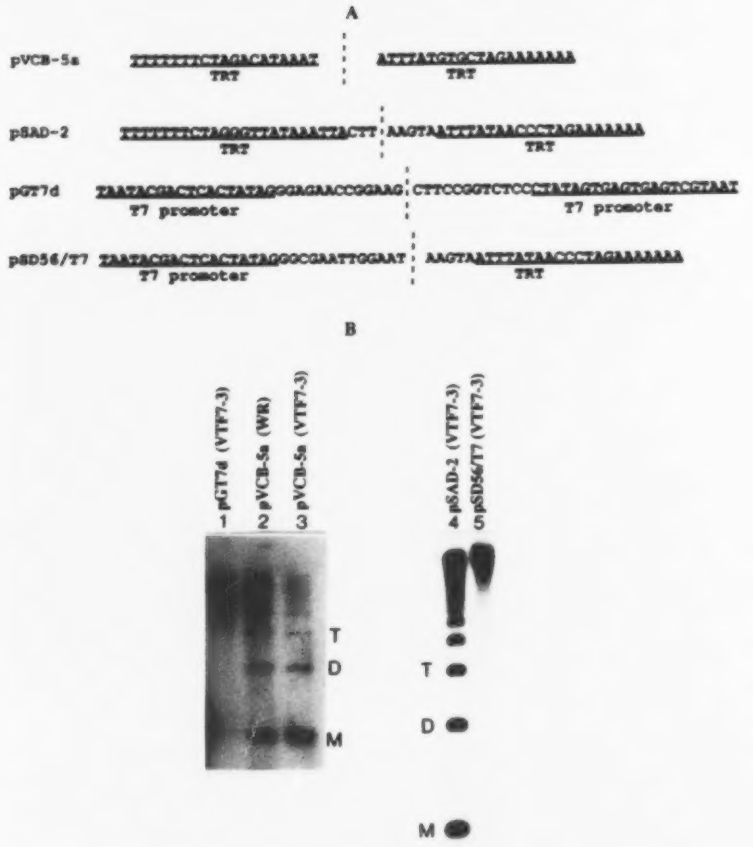
A
pVCB-5a
TTTTTTTCTAGACATAAAT
TRT
ATTTATGTGCTAGAAAAAAA
TRT
pSAD-2
TTTTTTTCTAGGGTTATAAATTACTT
TRT
AAGTAATTTATAACCCTAGAAAAAAA
TRT
pGT7d
TAATACGACTCACTATAGGGAGAACCGGAAG
T7 promoter
CTTCCGGTCTCCCTATAGTGAGTGAGTCGTAAT
T7 promoter
pSD56/T7
TAATACGACTCACTATAGGGCGAATTGGAAT
T7 promoter
AAGTAATTTATAACCCTAGAAAAAAA
TRT
B
1 pGT7d (VTF7-3)
2 pVCB-5a (WR)
3 pVCB-5a (VTF7-3)
T
D
M
4 pSAD-2 (VTF7-3)
5 pSD56/T7 (VTF7-3)
T
D
M

Figure IV-5. *In vivo* transcription is not sufficient to mediate inverted repeat resolution in poxvirus infected cells. The TRT sequences of the cloned replicative intermediates of vaccinia virus (pVCB-5a), and SFV (pSAD-2). An inverted repeat of the phage T7 promoter (pGT7d), and a construct that contains one TRT from SFV and one phage T7 promoter (pSD56/T7). The promoters in all of the plasmids are arranged so that transcription is directed toward the axis of symmetry which is indicated by the vertical dashed line. The central axis sequences of all of the constructs has perfect or near perfect inverted repeat symmetry. (B) Southern blot analysis of the plasmid DNA recovered after transfection into poxvirus infected cells. The name of the transfected plasmid is indicated above each lane and the virus that the cells were infected with is indicated in brackets WR= the wild type vaccinia virus WR strain. VTF7-3= a recombinant vaccinia virus that expresses the phage T7 RNA polymerase. Positions of the monomer (M), dimer (D), and trimer (T), linear minichromosomes are indicated.

the T7 promoter to direct the synthesis of RNA was tested by creating a fusion with the bacterial LacZ gene under the regulation of a phage T7 promoter. This construction was transfected into cells that had been infected with VTF7-3 resulting in the formation of blue plaques indicating that the T7 promoter sequence was functional and that the T7 RNA polymerase was being synthesized in an active form in the infected cells. We then tested the influence of transcription on an inverted repeat structure that was composed of one complete and functional copy of the TRT sequence from SFV and one deleted copy of the TRT. The construct pSD56/T7 has a phage T7 promoter replacing the deleted part of one copy of the SFV TRT sequence (Fig. IV-5A). This plasmid was replicated into a high molecular weight structure when transfected into cells that had been infected with either wild type vaccinia virus or with VTF7-3 but no resolved minichromosome species could be detected in either case (Fig. IV-5B, lane 5). Thus, transcription proceeding through the inverted repeat region was not sufficient to rescue resolution of inverted repeat constructs from which one TRT sequence had been deleted.

D. Discussion

At late times in the poxvirus replicative cycle most of the viral DNA exists in long concatemeric arrays that are joined end-to-end by a fusion of the viral hairpin telomere sequences. At the same time promoter sequences within the viral telomere region direct the synthesis of noncoding transcripts into and likely across the concatemer junctions of the replicative intermediates. The production of noncoding transcripts seems like a very unusual behavior for poxviruses which have genomes tightly packed with open reading frames and which strictly regulate their gene expression (Moss, 1990). The two promoters (TRT and cTRT) that direct transcription of the terminal region at late times are highly conserved in sequence and spatial orientation between the Leporipoxvirus SFV and the Orthopoxvirus vaccinia virus (Parsons & Pickup, 1990, Stuart *et al.*, 1991). The conservation of the terminal promoters suggests that they may play an important role in the virus replicative cycle. The use of cloned versions of the SFV telomere replicative intermediates in an *in vivo* transfection assay revealed that deletions of the TRT sequence or mutations that eliminated the promoter activity of the TRT also destroyed the ability of the TRT to function as a target sequence for the resolution of replicative intermediates (DeLange *et al.*, 1986, DeLange & McFadden, 1987, Stuart *et al.*, 1991). We have under taken a study of the TRT promoter elements and the role that transcription may play in the resolution of poxvirus replicative intermediates.

The poxvirus replicative cycle is a complex process that is dependent upon sequentially connected relationships among numerous aspects of viral nucleic acid metabolism (reviewed by Moss, 1990). Early gene expression is a prerequisite for viral DNA replication and all subsequent late events. The process of DNA synthesis is an essential feature of viral homologous recombination (Ball, 1987, Evans, *et al.*,1988, Merchlinsky, 1989); and the newly replicated viral DNA forms templates for the expression of the intermediate and late classes of viral genes (Keck *et al.*, 1990). Late viral gene products are in turn essential for the resolution of viral replicative intermediates into mature hairpin terminated genomes (Merchlinsky & Moss, 1989a).

Investigating the relationship between active transcription and replicative intermediate resolution has been difficult for several reasons. No specific inhibitors of the viral RNA polymerase are known, and the well characterized temperature-sensitive RNA polymerase mutants are defective in some aspect of their folding and assembly (Hooda-Dhingra *et al.*, 1989) and can not be rapidly inactivated at a specific period in the infectious cycle. Finally the RNA that is synthesized at late times is very unstable (Sebring & Salzman, 1967) and the vaccinia virus late polypeptides show a great variability in stability (Pennington, 1974). Thus inhibiting transcription may prevent telomere resolution by limiting the availability of some necessary gene product. By inhibiting transcription with an RNA chain terminator we hoped to determine what role, if any the viral RNA polymerase complex played in resolving replicative intermediate structures. The use of an RNA chain terminator also allowed us to distinguish between a requirement for active RNA synthesis and simple binding of the TRT promoter and unwinding of the DNA helix by late transcription factors The application of cordycepin to infected/transfected cell monolayers inhibits the resolution of pre-existing poxvirus replicative intermediates. A simple explanation of this result is that active transcription initiated from the TRT promoter and proceeding through the concatemer junction is in fact an essential feature of the resolution process. A corollary to this interpretation is that binding of the TRT promoter sequence by the viral late transcription factors and RNA polymerase complex is not sufficient to catalyze the resolution event. This explanation while appealing is confounded by the potential instability of some of the viral late gene products.

Deletions of the TRT sequence that affect only one side of the replicative intermediate are lethal to the resolution of the replicative intermediate (DeLange *et al.*, 1986). This observation initially lead us to the conclusion that the resolution of replicative intermediates required two functional late promoters that direct transcription toward one another. This suggestion was supported by the observation that symmetrical single nucleotide substitutions within the TRT that abrogate promoter activity similarly affect resolution (Stuart *et al.*, 1991 and Fig. IV-2). However, single nucleotide substitutions that eliminate promoter activity on only one side of the inverted repeat are not lethal to the resolution of replicative intermediates (Fig. IV-2), with respect to mutations made within the conserved ATTTA motif of cloned versions of the SFV replicative intermediate. It is not clear how the T-to-C substitution within the ATTTA motif inhibits transcription. It may be that this mutation prevents the initiation and elongation of a transcript but still allows the recruitment of RNA polymerase and the associated transcription complex formation. In this case a replicative intermediate bearing one wild type TRT sequence and one TRT with an T-to-C substitution would engage a transcription complex on each of the TRT copies and possibly be unwound. In this scenario, both TRT sequences could be unwound but only one of the complexes could proceed to transcribe the terminal sequences. We assume this to be the case with our single side TRT mutations since a mutation that eliminated the binding of the transcription complex entirely is predicted to behave identically to a deletion of the TRT.

The DNA helix of viral intermediate gene promoters is unwound by the formation of a transcription complex. This occurs through the action of a single transcription factor and can occur even under conditions that are not amenable to the elongation of a nascent transcript (Vos *et al.*, 1991a). Although three viral late transcription factors have been identified (Keck *et al.*, 1990), the mechanism of late transcript initiation remains poorly understood. Unwinding of late promoters by a viral transcription complex has not been demonstrated; however, it seems likely to occur and unwinding of the TRT sequence by a transcription complex might prove to be a critical event in the initiation of the strand-exchange that is required for the resolution of telomere replicative intermediates. It is also possible that some subunit of the transcription complex promotes DNA looping by the dimerization of two bound transcription complexes. In either case a site-specific nicking and strand-exchange event followed by branch migration could account for the formation of heteroduplex DNA at the extreme terminus of the hairpin (Merchlinsky, 1990). In either of these models transcription driven from one or both sides of the inverted repeat should be sufficient to provide the helicase activity necessary to propagate the strand-exchange and branch migration required in telomere resolution.

Viral early promoter sequences can not replace the TRT for its function in the resolution of an inverted repeat structure (Fig. IV-4). The SFV T1 early promoter and the vaccinia virus 7.5 kDa gene promoter fail to function in the resolution of an inverted repeat at early or late times or when DNA replication is allowed to proceed in the absence of late protein synthesis, (i.e. following infection with vaccinia virus ts 53 at the non-permissive temperature). Inverted repeat replicative intermediates are not normally present at early times during infection and there are three stop signals for early transcription present between the last open reading frame and the hairpin terminus of SFV. It may be important that transcription complexes not enter the terminal region at early times due to potential interference with early replicative activities that may be initiated near the hairpin termini. The transfected constructs that contained early promoters in an inverted repeat configuration were presumably being transcribed to some extent prior to the expression of the late gene products that are believed to be required for the resolution of replicative intermediates. In this case it is probable that transcription of the inverted repeat constructs was not occurring at the same time that late viral gene products were becoming available. In order to determine if resolution could be accomplished by the transcription of an inverted repeat structure in the presence of viral late gene products, the TRT sequence was replaced with a heterologous promoter from the bacteriophage T7. A construct containing an inverted repeat dimer of this promoter was replicated but not resolved when transfected into cells infected with either wild type vaccinia virus or with a recombinant vaccinia virus that expresses the bacteriophage T7 RNA polymerase (VTF7-3) (Fig. IV-4). This promoter construct was fully active for transcription at both early and late times as demonstrated by the ability of the promoter to drive the synthesis of β-galactosidase activity when transfected into cells that had been infected with VTF7-3.

The deletion of much of the TRT sequence on one side of the inverted repeat replicative intermediate is lethal to resolution and cannot be rescued by transcription with a heterologous RNA polymerase (Fig. IV-5). We can conclude from the above observations that active transcription initiated in a convergent fashion from either side of the inverted repeat is not sufficient to generate a resolution competent structure. Rather, it appears that the defined nucleotide sequence of the TRT is required not only for the initiation of transcription but for the binding of a transcription complex or specific transcription factor on both sides of the inverted repeat replicative intermediate. Another possibility is that a specific viral late "resolution factor" recognizes the TRT sequence and competes with the transcription complex for binding to this sequence. The transcription complex encoded by poxviruses is exceedingly complex, composed of 7 subunits and multiple associated proteins (Moss, 1990). It has been shown that some of the RNA polymerase accessory proteins are specific for the transcription of only the late class of genes (Keck, *et al.*, 1990). The thermolabile vaccinia virus ts53 encodes a mutation in the 147 kDa RNA polymerase subunit that renders the virus capable of transcribing early but not late genes when an infection is allowed to proceed at the non-permissive temperature (Hooda-Dhingra *et al.*, 1989). One interpretation of this observation is that the viral transcription complex that is recruited to and transcribes late promoters such as the TRT sequence may be considerably different in subunit composition from the RNA polymerase that transcribes early genes. However, another interpretation is that in the absence of nascent RNA polymerase subunit synthesis, the preexisting active enzyme complexes are diluted out over time within the cellular environment. Furthermore three different sets of viral transcription factors are expressed throughout the course of a productive infection. Both the early and intermediate transcription factors have a profound effect upon the configuration of the cognate promoter sequences to which they bind. These effects are manifest by unwinding the promoter sequence (Vos *et al.*, 1991) or by bending the DNA at the promoter (Broyles *et al.*, 1991). The effect of the late transcription factors upon promoters such as the TRT sequence is not yet known; however, bending or unwinding of DNA sequences that are arranged in an inverted repeat configuration could well provide the manipulation required to initiate replicative intermediate resolution.

The viral capping enzyme complex has been shown to be associated with the early RNA polymerase and to function directly in the termination of early viral transcripts in a sequence dependent fashion (Broyles & Moss, 1987, Luo *et al.*, 1991) The capping enzyme is also a required factor for the initiation of viral intermediate gene transcription (Vos *et al.*, 1991b). The capping enzyme complex has been implicated for involvement with telomere resolution by the identification of a temperature-sensitive vaccinia virus strain bearing a mutation in the small subunit of the capping enzyme (Carpenter & DeLange, 1991). This mutant virus appears to be proficient in late gene expression but is defective in telomere resolution (Carpenter & DeLange, 1991).

Two of the late promoter sequences that we tested, cTRT and vaccinia virus 11 kDa gene promoter, can functionally replace the TRT with regard to the resolution of inverted repeat sequences (Fig. IV-5). The vaccinia virus 11 kDa gene promoter is identical to the TRT sequence in the two regions that have been defined to be essential to the TRT for both promoter function and the resolution of replicative intermediates. The cTRT which is present upstream of the TRT in the SFV terminus can functionally replace the TRT in spite of its disparity in sequence (Stuart *et al.*, 1991). However; the cTRT functions only poorly and generates very low quantities of resolved products. The common feature of the TRT, cTRT and 11 kDa gene promoter sequences is that they are strong late promoters. However, another strong late promoter derived from the cowpoxvirus A type inclusion body (pATI) is not able to functionally replace the TRT sequence in spite of possessing the conserved TTTTTTT box I and the TAAAT box Ia motifs. The major factor influencing the inability of the ATI promoter to mediate telomere resolution may be the space between the two conserved sequence motifs. The TRT sequences from SFV, vaccinia virus, cowpoxvirus, and fowlpoxvirus all have the box I and Ia sequences separated by a sequence nonspecific spacer region consisting of 9-11 nucleotides (Merchlinsky, 1990). In the case of the ATI promoter the conserved sequences are separated by only 4 bp. The sequence of the spacer region has been shown to be nonessential for telomere resolution (Merchlinsky & Moss, 1989b), and may act only to separate the two sequences such that essential protein-protein contacts can be made between resolution factors. The failure of the ATI promoter to mediate inverted repeat resolution clearly implies that resolution requires a complex interaction between the TRT nucleotide sequence and some specific resolution factors that either reside with the RNA polymerase complex or compete for binding to the TRT. It is still likely that the transcriptional complex plays some role in resolution either by targeting the resolution factors to the TRT sequence or by providing a helicase activity to unwind the DNA and promote strand-exchange. However, it is clear that transcription of the inverted repeat is in itself insufficient to mediate telomere resolution.

The utilization of a promoter element and specific transcription to resolve replicative intermediates is an interesting, but not entirely novel example of viruses using a sequence element for multiple functions (DePamphilis, 1988). Promoter sequences and transcriptional enhancers are known to be required for the initiation of DNA replication in papovaviruses, adenovirus and papillomaviruses (Hassel *et al.*, 1986, DePamphilis, 1988). Parvoviruses contain a transcriptional enhancer element within the terminal hairpin sequence that is known to be important for replication initiation, the resolution of viral replicative intermediates and virion packaging (Liu *et al.*, 1991). Papillomavirus DNA replication is dependent upon the complex formed between the initiator protein E1 and the transcriptional activator E2. The formation of a complex between the two proteins allows the replication initiator protein to be targeted to the origin of replication which also serves as the activator binding site (Mohr *et al.*, 1990, Yang *et al.*, 1991), Interestingly, in the papilloma virus case, replication activity was not inhibited *in vitro* by α-amanitin (Yang *et al.*, 1991). Thus, although transcription factor binding was required to initiate replication, active transcription was dispensable. Transcription has been shown to alter the topological state of the transcribed sequences (Giaever & Wang, 1988, Liu & Wang, 1987) and can stimulate homologous recombination as in the case of yeast (Thomas & Rothstein, 1989), and in the mammalian immunoglobulin gene locus (Schlissel & Baltimore, 1989).

The bacteriophage T7 utilizes a specific transcription event to resolve the concatemers of genomes generated by the replication and recombination of its DNA (Chung & Hinkle, 1990b). Resolution of the concatemers of phage T7 DNA requires multiple gene products and sequence elements as well as orientation specific transcription through the concatemer junction (Chung & Hinkle, 1990a). Phage promoters other than the one that normally directs transcripts through the concatemer junction will suffice for the resolution of concatemers so long as the transcript is directed in the correct orientation (Chung & Hinkle, 1990a, 1990b). In the case of phage T7 the possibility that the transcript generated from the concatemer junction sequence is catalytic cannot be formally excluded. Cloned poxvirus replicative intermediates that have intact TRT sequences can be resolved even when most of the DNA sequence between the two TRT sequences has been deleted. Thus if a catalytic RNA molecule were generated from the TRT it would have to be capable of interacting with transfected DNA molecules that lack its homologous sequences.

Drugs that inhibit late transcription such as cordycepin cause a defect in telomere resolution. This could be due directly to the inhibition of transcriptional elongation or to the requirement for limiting amounts of a labile late gene product. A vaccinia virus mutant with an abortive late phenotype that transcribes late genes but rapidly degrades its own RNA is also defective in telomere resolution (Merchlinsky & Moss, 1989a, DeLange, 1989). This further indicates that the transcription of the TRT sequences alone is not sufficient to catalyze the resolution of replicative intermediates. Without an *in vitro* resolution system composed of purified or semi-purified proteins we can only infer that the viral transcriptional apparatus is directly involved in the resolution of replicative intermediates. To date no TRT specific binding factors have been identified that might be part of a transcription complex or might compete with the transcription complex for the TRT sequence. The TRT sequence corresponds to one of the strongest naturally occurring viral promoters and it is reasonable to expect that transcription complexes would be recruited to this sequence. Further investigation should reveal the presence of TRT specific binding factors and the ability of these factors to promote either transcription or replicative intermediate resolution.

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Chapter V

In Vitro Resolution of Poxvirus Replicative Intermediates into Linear Minichromosomes with Hairpin Termini by a Virally Induced Holliday Junction Endonuclease

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A. Introduction

Poxviruses such as vaccinia virus and Shope fibroma virus (SFV) replicate exclusively in the cytoplasm of infected host cells and are believed to encode most, if not all, of the gene products required for their replicative cycle (Moss, 1990). The poxviral DNA genomes that have been studied are linear double stranded molecules that possess covalently closed hairpin termini (DeLange *et al.*, 1986, Geshelin & Berns, 1974) and have coding capacities for over two hundred gene products (Goebel *et al.*, 1990). The cytoplasmic site of infection and apparent ability to replicate in enucleated cells (Prescott *et al.*, 1971) suggests that all of the enzymatic activities required for poxviral DNA replication are virally encoded (reviewed in Traktman, 1990). In addition to enzymes required for DNA precursor biosynthesis, such as thymidine kinase (Hruby & Ball, 1982), thymidylate kinase (Smith *et al.*, 1989a) and ribonucleotide reductase (Slabaugh *et al.*, 1984, Slabaugh *et al.*, 1988), poxviruses have been found to encode and express genes for DNA polymerase (Jones & Moss, 1984, Magee & Miller, 1967), DNA ligase (Smith *et al.*, 1989b, Spadari, 1976), topoisomerase (Shaffer & Traktman, 1987, Shuman & Moss, 1987) and a number of nucleases (Lakritz *et al.*, 1985, Pogo & Dales, 1969, Rosemond-Hornbeak *et al.*, 1974). It has been shown through genetic and molecular biological means that poxvirus DNA is subject to extensive genetic recombination during its replicative cycle (Ball, 1987, Fenner & Comben, 1959, Gemmel & Cairns, 1959, Spyropoulos *et al.*, 1988). Efficient recombination has also been observed between coinfecting viral genomes (Fenner & Comben, 1959), and between viral genomic DNA and homologous sequences in transfected plasmid DNA (Nakano *et al.*, 1982). This observation has proven useful for the mapping of viral genes (Weir *et al.*, 1982) and for the construction of recombinant poxviruses for use as vectors (Mackett *et al.*, 1982). The induced recombination activity is not specific for viral DNA sequences because unrelated co-transfected bacterial plasmids are subject to both intermolecular and intramolecular recombination in poxvirus infected cells (Evans *et al.*, 1988). Analysis of the viral products of recombination have demonstrated that both single and double cross over events occur with high frequency (Ball, 1987, Spyropoulos *et al.*, 1988) and the unstable tandem repeat products of single crossover events are rapidly processed via second crossover events into more stable structures (Spyropoulos *et al.*, 1988).

The induction of recombination activity in poxvirus-infected cells shows a stringent requirement for viral DNA replication. Cells infected with poxvirus mutants that are conditional lethal for DNA replication are unable to catalyze intramolecular recombination at the non-permissive temperature (Merchlinsky, 1989). Similarly, infected cells that have been treated with inhibitors of viral DNA replication are unable to recombine transfected plasmids (Evans *et al.*, 1988) or tandem repeats within the genomic DNA (Ball, 1987). It has thus been impossible to date to unlink the processes of DNA replication and recombination in poxvirus-infected cells. However, mutant poxviruses that are conditionally lethal for late protein synthesis but competent for DNA replication are capable of recombining transfected DNA (Merchlinsky, 1989) suggesting that all of the enzymatic activities required for recombination are synthesized at early times during infection. The autonomous nature of the poxvirus replicative cycle makes it likely that the observed recombination activities are catalyzed by virally encoded enzymes although none of the gene products responsible have yet been identified.

The replication of poxvirus DNA leads to the formation of transient high molecular weight concatemers of viral DNA (DeLange, 1989, Merchlinsky & Moss, 1989, Moyer & Graves, 1981). The precise structure of these replicative intermediates has not been completely characterized but it is clear that most, if not all, of the DNA exists as a linear array (DeLange, 1989, Merchlinsky & Moss, 1989). Although the presence of branched or circular intermediates has not been excluded little evidence exists to verify the occurrence of such structures. The arrays of viral DNA are joined end to end such that the junction between genomes exists in the form of an inverted repeat (DeLange & McFadden, 1990). It has been shown that a cloned version of this inverted repeat can be extruded in a cruciform configuration *in vitro* if the purified plasmid is subjected to negative superhelical torsion (DeLange *et al.*, 1986, Dickie *et al.*, 1987, Merchlinsky *et al.*, 1988). At its base an extruded cruciform is structurally identical to a Holliday junction, which is a key intermediate in most models of general genetic recombination (Holliday, 1964). The final stage in homologous recombination requires the cleavage of a Holliday junction and the subsequent ligation of the newly generated recombinant duplexes. Endonucleases that display specificity for Holliday junctions and branched DNA structures have been isolated from phage T4 (Kemper & Garabet, 1981), phage T7 (deMassey *et al.*, 1987), S. cerevisiae (Evans & Kolodner, 1988, Jensch *et al.*, 1989, Symington & Kolodner, 1985, West & Korner, 1985), human placenta (Jayaseelan & Shanmugam, 1988), HeLa cells (Waldman & Liskay, 1988) and calf thymus (Elborough & West, 1990).The function of these endonucleases is relatively well established in the case of the bacteriophages. At late times in the replicative cycle phage T4 primes replication by strand invasion with the concomitant formation of branched DNA structures (Mosig, 1983). Maturation and packaging of replicated DNA requires the resolution of these branched intermediates into unit sized progeny genomes (Frankel *et al.*, 1971, Jensch & Kemper, 1986). The replication of phage T7 DNA results in the formation of long concatemeric structures that must be cleaved in order to be packaged (Stratling *et al.*, 1973, Studier, 1969). Both phage T4 and T7 undergo homologous recombination at high frequency (Doermann & Hill, 1953, Studier, 1969) and phage mutants defective in these resolvase activities are deficient in homologous recombination (Kerr & Sadowski, 1975, Powling & Knippers, 1974). At the non-permissive condition these mutants accumulate branched recombination intermediates (Tsujimoto & Ogawa, 1978) and are deficient in maturation and packaging of replicated DNA (Paetkau *et al.*, 1977, Stratling *et al.*, 1973). No clear role has been assigned to the enzymes in yeast although it is proposed that they serve some function in recombination (Jensch *et al.*, 1989).

Vaccinia virus encodes several nucleases one of which is capable of cleaving single stranded DNA and ligating some of the cleavage products (Lakritz *et al.*, 1985, Merchlinsky *et al.*, 1988, Reddy & Bauer, 1989). This enzyme, designated nicking-joining enzyme, cleaves branched DNA structures at single stranded regions but displays no specificity for the base of the branched substrate, thus its role in viral replication or recombination remains to be clarified.

Poxviral telomere resolution bears important similarities to site specific recombination (reviewed in DeLange & McFadden, 1990) and hence elucidation of the mechanism of the resolution of concatemeric arrays of viral DNA into hairpin terminated daughter molecules may shed light on the biochemistry of site specific recombination as well. In an attempt to develop an *in vitro* model for the resolution of poxvirus replicative intermediates we have identified an endonuclease activity induced at late times in poxvirus infected-cells that displays specificity for the branched DNA structures of extruded cruciforms. This endonuclease activity resolves cloned versions of the SFV replicative intermediate *in vitro* to yield hairpin terminated minichromosomes that are indistinguishable from those generated *in vivo*. In addition, asymmetric nicks are observed within the viral telomere resolution target sequence (TRT), suggesting that some of the specificity of resolution *in vivo* can be reproduced by these extracts *in vitro* .

B. Materials and methods

Cells and viruses. BGMK cells (African green monkey kidney) obtained from the ATCC were propagated as monolayer cultures in Dulbeco's modified Eagle medium supplemented with 10% newborn calf serum (Gibco Laboratories). HeLa S3 cells, provided by Dr. V. Paetkau, were maintained as suspension cultures in Joklik modified

Eagle's medium (Flow Laboratories) supplemented with 10% equine serum (Gibco Laboratories). Vaccinia virus (WR) and SFV (Kasza) were obtained from the ATCC. Viruses were propagated by infection of BGMK monolayers (SFV) or HeLa suspension cultures (vaccinia virus) at a multiplicity of one infectious unit per cell. Virus stocks from these infected cells were generated as described previously (Condit & Motyczka, 1981, Wills *et al.*, 1983). The vaccinia virus temperature sensitive mutant ts53 was generously provided by Dr. R. Condit. The conditions for growth and infection of this mutant have been previously described (Condit & Motyczka, 1981).

Plasmids and Strains. Bacterial plasmids that contain inverted repeats were propagated in the recombination deficient Escherichia coli strain DB1256 (DeLange *et al.*, 1986), and all other plasmids were maintained in E. coli HB101. The plasmid pAT34, provided by Dr. David Pulleyblank, contains an insert of the self-complementary alternating purine/pyrimidine sequence d(AT)n-d(AT)n (Haniford & Pulleyblank, 1985). The construction and purification of plasmids that carry inverted repeats of SFV telomere sequence pSCB1a, and pSAD-2 has been described (DeLange *et al.*, 1986). Variants of the SFV telomere sequence generated by site specific mutagenesis have been described previously (Stuart *et al.*, 1991).

Preparation of cell lysates. Cytoplasmic or whole cell lysates were prepared by a modification of the procedure for preparing nuclear extracts competent for *in vitro* transcription (Manley & Gefter, 1981). HeLa S3 cells, 3 to 6 litres grown to a density of 3-5 x10^5 cells per ml, were concentrated by centrifugation to 5x10^6 cells per ml and infected with vaccinia virus at a multiplicity of 5-10 infectious units per cell. Following a one hour adsorption the cells were diluted 10 fold and the infection was allowed to proceed for 14-16 hours. The infected cells were harvested by centrifugation, washed with ice cold 0.01 M phosphate buffered saline pH 7.4 (PBS) and resuspended in 4 packed cell volumes (PCVs) of ice cold 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5 mM DTT. After swelling for 15 min on ice the cells were broken by 20 strokes of a Dounce homogenizer. A cytoplasmic fraction was obtained by pelleting the nuclei and cell debris for 10 min at 600 x g. These cytoplasms or whole cell lysates were then mixed with 4 PCVs of 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose and 50% glycerol. After 10 min of gentle stirring at 4°C the salt concentration was raised by the addition of 1 PCV of a saturated $(NH_4)_2SO_4$ solution which also served to lyse the nuclei in whole cell extracts. This homogenate was stirred for 20 min at 4°C and then chromatin and cell debris were removed by centrifugation at 47,500 rpm for 4 hours at 4°C in a Beckman Ti70 fixed angle rotor. Supernatants from the high speed spin were precipitated by the addition of 0.35 grams of solid $(NH_4)_2SO_4$ per ml of homogenate and neutralized with 10 µl of 1 N NaOH per gram $(NH_4)_2SO_4$. The precipitate was pelleted by centrifugation at 15000 x g for 30 min in a Sorvall SS34 rotor at 4°C and resuspended in 1/10 the volume of the original high speed supernatant in buffer A: 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 0.01% NP-40, 2 mM DTT and 10% glycerol. The suspension was dialyzed against 100 volumes of the buffer A for 10 hours with two changes of buffer. This preparation was assayed for activity and then the KCl concentration was increased to 250 mM and the homogenate was chromatographed on DEAE-Sepharose. The flow through fraction was again dialyzed against buffer A for 6 hours and then was concentrated by ultrafiltration in Centricon 10 concentrators (Amicon). This preparation was then aliquoted, snap frozen in a dry ice-ethanol bath and stored at -70°C. In some cases the infection was performed in the presence of 40 µg/ml cytosine-β-D-arabinofuranoside (araC) to inhibit viral DNA replication and late gene expression. When the cell lysates were made following infection with vaccinia virus ts53 the virus adsorption was for 2 hours and the infection was allowed to proceed at either the permissive temperature 32°C or the non-permissive temperature 39.5°C.

Enzymes and drugs. DNA restriction and modification enzymes used in the construction of plasmids were obtained from Bethesda Research Laboratories and used as directed by the manufacturer. Topoisomerase I purified from calf thymus was a generous gift from Dr. A. R. Morgan. Topoisomerase I was used for the relaxation of plasmid DNA specifically to resorb cruciforms that were extruded by negatively supercoiled plasmid DNA. Relaxation of plasmids was accomplished by incubating plasmid DNA with 100 μg/ml ethidium bromide on ice in darkness. Following a 15 hour incubation the mixture was made to 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and extracted 6 times with ice cold isobutanol saturated with 50 mM Tris-HCl (pH 7.6) and once with ice cold chloroform. DNA was then relaxed by the addition of 5 units of calf thymus topoisomerase I and the reaction was allowed to warm slowly to room temperature at which time one unit of topoisomerase was added and the reaction was allowed to proceed for 30 min at 37°C. Topoisomerase and salts were removed by extraction with phenol and chloroform followed by dialysis against 10 mM Tris-HCl (pH 8.0). Phage T7 gene 3 endonuclease was purified from the E. coli strain W3110 harbouring pAR2510 (provided by Dr. W. Studier) which carries the T7 gene 3 under the control of the LacUV promoter inserted into the plasmid pBR322 (deMassey *et al.*, 1987). Expression of this gene is induced by the addition of isopropylthio-β-D-galactoside (IPTG) to 0.4 mM to a culture of exponentially growing cells at a density of $5x10^8$ cells/ml. Cleared cell lysates were prepared as described (deMassey *et al.*, 1987). The lysate was applied to an 8 ml phosphocellulose column equilibrated with 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM β-mercaptoethanol and 4% glycerol (buffer 1). Gene 3 endonuclease was step eluted with 0.6 M NH_4Cl in buffer 1 (Fraction 2). The phosphocellulose fraction was diluted with 3 volumes of buffer 1 and loaded on to a 4 ml column of CM52 cellulose equilibrated with 0.15 M NH_4Cl. The column was then developed with a 0.15-0.6 M NH_4Cl gradient. Fractions with gene 3 endonuclease activity which eluted at 0.3 M NH_4Cl (fraction 3), were diluted with 2 volumes of buffer 1 and applied to a 0.2 ml column of Biogel HTP DNA grade hydroxyapatite equilibrated with 0.1 M NH_4Cl in buffer 1. The peak fractions of gene 3 endonuclease collected from a 0.1-1.0 M NH_4Cl gradient were pooled, concentrated against polyethylene glycol (8000) and dialyzed against 50 mM Tris-

HCl (pH 8.0), 1 mM DTT, 50% glycerol (HTP pool). The activity of the T7 endonuclease was assayed in 20 μl reactions containing 1 μg target DNA, 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 4 mM spermidine, 50 μg/ml denatured gelatin, and cleavage reactions were conducted for 5 min at 37°C. Specific endonuclease activity in vaccinia virus-infected cell lysates was assayed in 50 μl reactions containing 1 μg target DNA, 50 mM Tris-HCl (pH 8.0), 100 mM KCl or potassium glutamate, 0.1 mM EDTA, 0.01% NP-40, 2 mM DTT, 10% glycerol, 2 mM $MgCl_2$, 1 mM ATP, for 15 min at 30°C. DNA cleavage assays were stopped by the addition of 0.5% SDS, 20 mM EDTA and 2 mg/ml proteinase K (Sigma). The products of this assay were extracted with phenol and chloroform and then ethanol precipitated. The resuspended DNA was then cleaved with restriction enzymes to identify the sites of cleavage, electrophoresed through a 0.7% agarose gel and stained with ethidium bromide. Specific nicks created in substrate DNAs by the infected cell lysate were mapped by cleaving the nicked DNA with *Hind*III and then 3' end-labelling the fragments using [α-^{32}P] dATP and Klenow fragment or 5' end-labelling with [γ-^{32}P] ATP and polynucleotide kinase. These labelled fragments were boiled and electrophoresed through 0.4 mm 8% polyacrylamide gels in the presence of 7 M urea. In some cases the labelled hairpin fragment was purified by electrophoresis through an 8% nondenaturing polyacrylamide gel. The labelled fragment was detected by autoradiography, excised as a gel slice, eluted by diffusion into 10 mM Tris-HCl (pH 8.0) and ethanol precipitated. The purified fragment was then analyzed by electrophoresis through 0.4 mm 8% polyacrylamide gels, in the presence of 7 M urea. Chemical sequencing was performed as described (Maxam & Gilbert, 1980) using chemicals and [α-^{32}P] dATP obtained from New England Nuclear Research Products.

Determination of hairpin structures. Hairpin terminated structures among the cleavage products generated by the infected cell lysate were detected by stopping the cleavage reactions with 0.2% SDS, 20 mM EDTA and 2 mg/ml proteinase K. Following a two hour digestion at 37°C the DNA was extracted with phenol:chloroform (1:1) and chloroform, then precipitated with ethanol and washed with 70% ethanol. DNA was resuspended and digested with 5 units of *Hind*III for one hour at 37°C. The products of this cleavage were boiled for 5 minutes and then quick chilled by immersing the tubes in ice water followed by electrophoresis through nondenaturing agarose gels. Apyrase, araC, diminazene aceturate, actinomycin D and cordycepin triphosphate were all obtained from Sigma Corp. *In vivo* assays for the resolution of SFV replicative intermediates in infected/transfected cells were performed as described (DeLange *et al.*, 1986).

C. Results

Assays for the resolution of viral replicative intermediates. To probe poxvirus-infected cell lysates for activities involved in the resolution of viral replicative intermediates, we utilized SFV-derived plasmid substrates that have previously been reported for *in vivo* resolution assays (DeLange *et al.*, 1986, Stuart *et al.*, 1991). Following transfection into infected cells, cloned poxvirus replicative intermediates are replicated initially into high molecular weight *Dpn*I-resistant concatemer structures (DeLange *et al.*, 1986, Merchlinsky & Moss, 1989). Resolution of these concatemer structures *in vivo* by trans-acting viral factors yields a ladder of replicated hairpin terminated linear minichromosomes. Cleavage of the fully and partially resolved linear molecules at a unique *Hind*III site produces molecules that during non-denaturing gel electrophoresis migrate as unit length linear plasmid DNA, a faster migrating fragment that has had one of its hairpin termini cleaved off and a very rapidly migrating hairpin molecule. We have mimicked this assay *in vitro* by utilizing the plasmid pSCB1a, which contains 1.5 kb of SFV telomere sequences as a substrate (Fig. V-1A). When plasmids that contain large inverted repeats are subjected to superhelical torsion the inverted repeat can be extruded into a cruciform structure (Fig. V-1A). Correct resolution of this plasmid by activities in an infected cell lysate would produce a linear hairpin terminated minichromosome species (Fig. V-1B). Cleavage of this linear

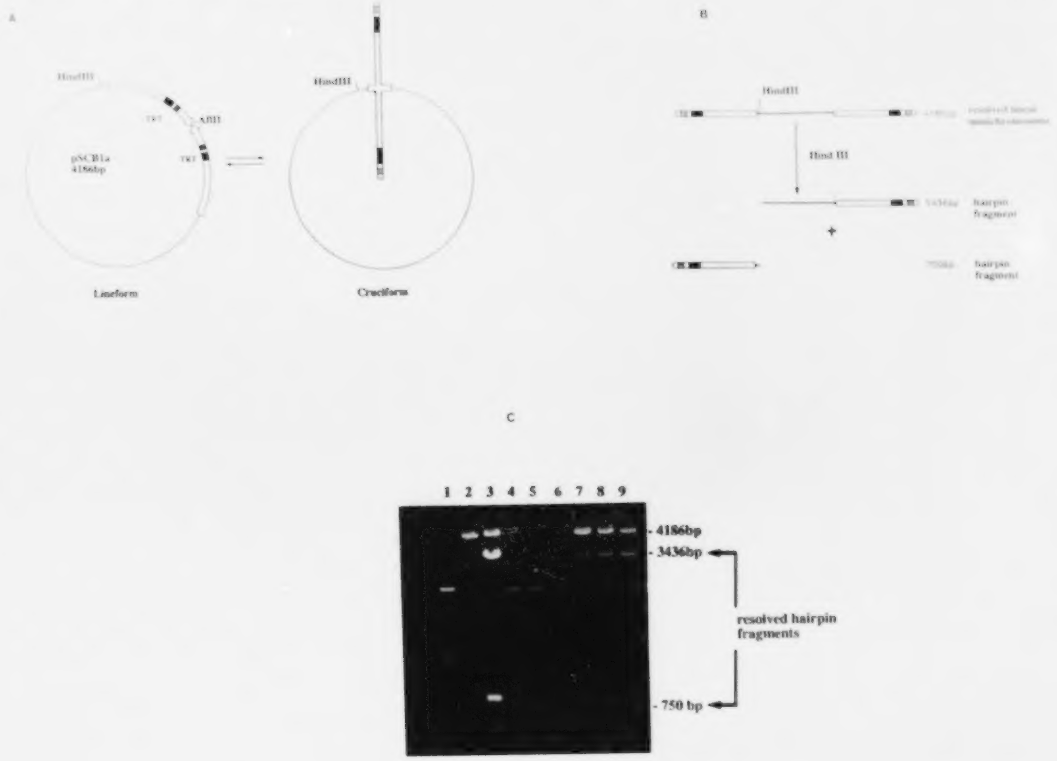

A
HindIII
TRT
AflII
pSCB1a
4186bp
TRT
Lineform
HindIII
Cruciform
B
HindIII
resolved linear minichromosome
Hind III
hairpin fragment
+
hairpin fragment
C
1 2 3 4 5 6 7 8 9
4186bp
3436bp
resolved hairpin fragments
750 bp

Figure V-1. Schematic representation of the plasmid substrate and products of the resolution assay. (A) the substrate pSCB1a is a pUC19 derived plasmid carrying a 1.5 kb inverted repeat version of a replicative form SFV telomere (DeLange *et al.*, 1986). The axis of dyad symmetry in the inverted repeat is denoted by the recognition site for the restriction enzyme *Afl*II. The telomere resolution target (TRT) sequences required for resolution of the inverted repeat into progeny hairpin termini are indicated by the stippled and shaded boxes within the inverted repeat. The lineform and extruded cruciform configurations of pSCB1a are shown. The pUC vector sequence is shown as a single line and the SFV insert is shown as a double line. (B) The linear hairpin terminated minichromosome that results from resolution of the plasmid pSCB1a. Cleavage of the linear plasmid at the unique *Hind*III site gives diagnostic fragments of 3436 bp and 750 bp. (C) An ethidium bromide stained gel showing the resolving activity present in the infected cell lysate. Lane 1: pUC19 linearized with *Hind*III, lane 2: pSCB1a linearized with *Hind*III. lane 3: pSCB1a cut with *Hind*III and *Afl*II to generate marker fragments. A portion of the 4186 bp linear fragment is resistant to digestion with *Afl*II due to the persistence of the cruciform structure in the substrate plasmid. Lanes 4, 5, and 6: pUC19 reacted with the infected cell cytoplasmic lysate at 30°C for 5, 15, and 30 minutes, respectively and then cleaved with *Hind*III. Lanes 7, 8, and 9: pSCB1a reacted with an infected cell cytoplasmic lysate at 30°C for 5, 15, and 30 minutes respectively, and then cleaved with *Hind*III.

plasmid with *Hind*III would yield a 3436 bp fragment with one hairpin end plus a 750 bp self-complementary hairpin molecule (Fig. V-1B). Relaxation of the circular template DNA by topoisomerase or random nicking would result only in linearization of the plasmid by *Hind*III. Similarly, random double-strand breaks induced by sequence non-specific endonucleases *in vitro* would lead to a variety of cleavage products that would appear as a smeared pattern following electrophoresis. Only a site or sequence specific cleavage of the plasmid could yield the correct single hairpin terminated 3.4 kb and 0.75 kb fragments following cleavage by *Hind*III.

Infected cell lysates resolve cloned replicative intermediates. Preliminary experiments using cleared cytoplasmic lysates from poxvirus-infected cells to resolve viral telomeric plasmids *in vitro* met with limited success, in part due to the high level of nonspecific viral-induced nuclease activities. However, the recent observation that the telomere resolution target (TRT) sequence is also a strong late promoter suggested that resolution and the late transcriptional machinery may be linked (Stuart *et al.*, 1991) and hence protein extracts enriched for viral late transcription factors were prepared and tested for resolution *in vitro*. A cytoplasmic extract generated from 6 litres of HeLa cells that had been infected with vaccinia virus for 15 hours was used initially to test for resolving activities.

Since the resolution targets of vaccinia and SFV are conserved and these viruses can efficiently resolve the others telomeres (DeLange *et al.*, 1986), the plasmid substrates used for the assays described below were from our library of SFV-derived constructs. The result of treating pSCB1a with this lysate for various periods of time followed by cleavage with *Hind*III is illustrated in Fig. V-1C. After a 5 minute incubation two discrete fragments of 3.4 kb and 0.75 kb are visible and these increase in intensity after incubation for 15 and 30 minutes (Fig. V-1C, lanes 7, 8, 9). Little random degradation of the plasmid DNA appears to occur over the course of the assay although the persistence of a portion of the full length linearized plasmid DNA indicates that not all of the substrate was cleaved. A plasmid DNA (pUC 19) that carries no viral sequence or large inverted repeats was rapidly relaxed by virally induced topoisomerase activity in the lysate (not shown) but no discrete cleavage products or random degradation were detected following treatment with *Hind*III (Fig. V-1C, lanes 4, 5, 6).

The specific resolving activity that we observed in our initial experiment was not detectable in lysates made in parallel with identically prepared cultures of uninfected cells (Fig. V-2, lane 3) or in cultures that had been infected with vaccinia virus for only 3 hours (Fig. V-2, lane 4). Plasmid DNAs were resolved into linear monomers by lysates from cells infected for 16 hours (Fig. V-2, lane 6) but not by lysates from cells that had been infected with vaccinia virus for 16 hours in the presence of the viral DNA replication inhibitor ara C (Fig V-2. lane 5). Lysates made from cells that had been infected with the thermolabile vaccinia virus mutant ts53 were active for the resolution of cloned replicative intermediates if the infection was allowed to proceed at the permissive temperature of 32°C but no activity was detectable in lysates that were made from cells infected at the non-permissive temperature of 40°C (data not shown). Since ts53 is defective in late gene expression due to a thermolabile RNA polymerase subunit (Thompson *et al.*, 1989) we conclude that late gene products must at least contribute to the resolution activity observed. Lysates made from two other cell lines (BGMK, SIRC) infected with vaccinia virus or SFV were found to contain too much endogenous nuclease activity to accurately monitor the virus specific resolving enzyme by this extraction procedure. These observations indicate that the resolving activity is poxvirus specific, is induced following the initiation of DNA replication and is dependent on late protein synthesis.

Characteristics of the resolving activity. The activity identified in poxvirus infected cell lysates cleaves plasmid DNA in a fashion indistinguishable from the resolution of cloned inverted repeats in the cruciform configuration by the phage T7 gene 3 endonuclease. Cleavage of the cruciform extruded by pSCB1a with the T7 endonuclease also generates a linear minichromosome molecule (Fig. V-3, lane 4). When this linearized

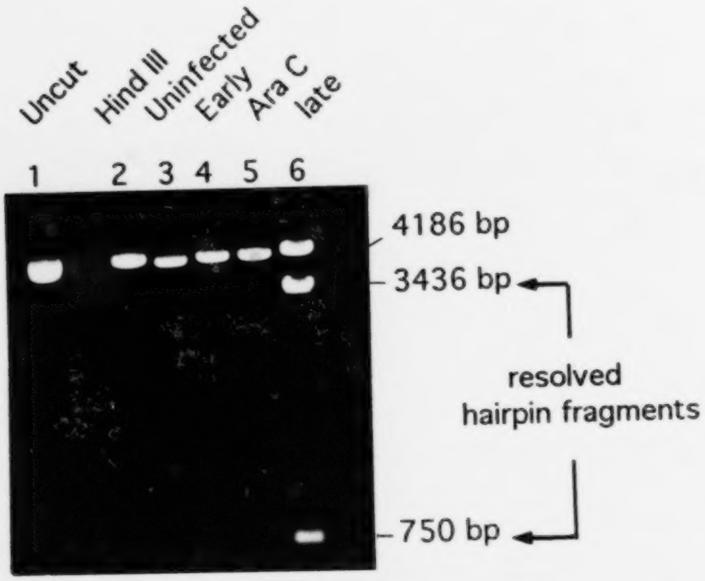

Uncut
Hind III
Uninfected
Early
Ara C
late
1
2
3
4
5
6
4186 bp
3436 bp
resolved
hairpin fragments
750 bp

Figure V-2. An ethidium bromide stained 0.7% agarose gel showing the resolution of the substrate plasmid pSCB1a by a vaccinia virus infected cell lysate. Lane 1: uncut plasmid DNA. lane 2: plasmid DNA linearized with *Hind*III. lane 3: plasmid DNA reacted with an uninfected cell lysate at 30°C for 30 mins., lane 4: plasmid DNA reacted at 30°C for 30 mins with a lysate made from cells that had been infected for 3 hours, lane 5: plasmid DNA reacted at 30°C for 30 mins with a lysate made from cells that had been infected for 16 hours in the presence of ara C, lane 6: plasmid DNA reacted for 30 mins with lysate made from cells that had been infected for 16 hours. The position of the resolved hairpin fragments is indicated by the arrows.

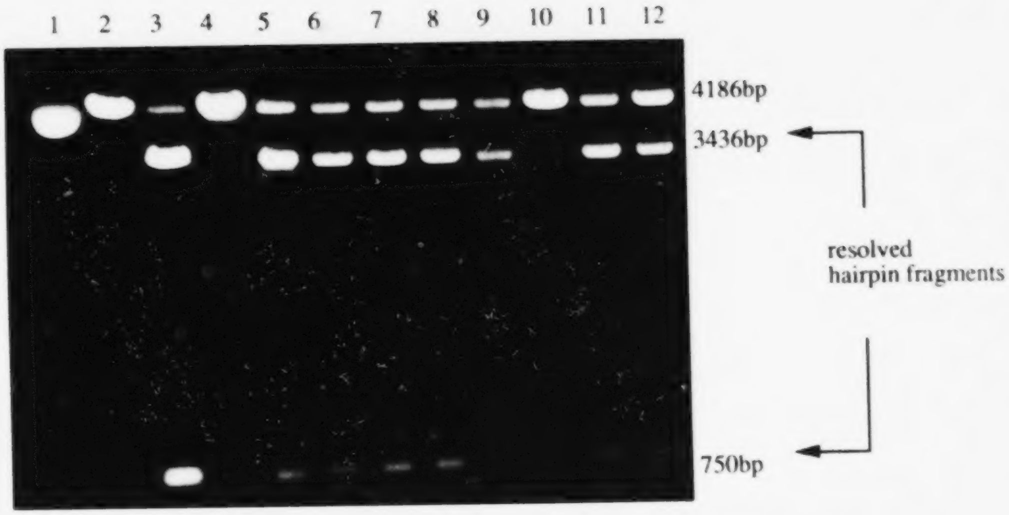


Figure V-3. Characterization of the resolvase activity in infected cell lysates with the plasmid pSCB1a. All reactions were carried out for 30 mins at 30^{o}C, the products were fractionated on a 0.7% agarose gel and visualized by staining with ethidium bromide. Lane 1: uncut plasmid. Lane 2: *Hind*III linearized standard. Lane 3: plasmid cleaved with *Hind*III and *Afl*II. Lane 4: plasmid cleaved with the phage T7 gene 3 endonuclease. Lane 5: plasmid digested with phage T7 gene 3 endonuclease and then cleaved with *Hind*III. Lane 6: plasmid reacted with lysate and then *Hind*III. Lane 7: deoxyribonucleotides omitted from the standard reaction. Lane 8: ribonucleotides,and deoxyribonucleotides both omitted from the reaction. Lane 9: ATP, ribonucleotides, deoxyribonucleotides all omitted from the reaction. Lane 10: $MgCl_2$, ATP, ribonucleotides, deoxyribonucleotides all omitted from the reaction and EDTA was added to 5 mM. Lane 11: Complete reaction mix. Lane 12: Complete reaction mix with 100 μM of the poxviral topoisomerase inhibitor diminazene aceturate added.

molecule is cleaved with *Hind*III discrete 3436 bp and 750 bp molecules are produced (Fig. V-3, lane 5) that are identical to those produced by digestion with infected cell lysate followed by *Hind*III (Fig. V-3 lane 6). The requirements for the lysate to accurately resolve the inverted repeat plasmid were also investigated (Fig. V-3). The initial complete reaction used to screen for resolution activity contained dATP, dTTP, dCTP, dGTP, ATP, TTP, CTP, GTP as well as 2 mM $MgCl_2$. Upon identification of the activity, however, we observed that the plasmids are efficiently resolved without the addition of dNTPs (Fig. V-3 lane 7) or NTPs (Fig. V-3, lane 8) or ATP (Fig. V-3, lane 9). Clearing the lysate of endogenous ATP with apyrase also had only minimal effect (data not shown). Although ATP does not appear to be strictly required its presence does stimulate the reaction. The chain terminating ATP analog cordycepin triphosphate does not stimulate the activity but has no inhibitory effect (data not shown), suggesting that RNA polymerization *per se* is not necessary for this resolution activity. Similarly, the addition of RNase A and T1 has no effect upon resolution of the plasmid substrate into linear minichromosomes (data not shown). A divalent metal ion is required because the chelation of Mg^{++} with EDTA abrogates the reaction (Fig. V-3, lane 10). The need for Mg^{++} is not stringent, however, and $MnCl_2$ can substitute for $MgCl_2$ (data not shown). Higher concentrations of $MgCl_2$ or the addition of spermidine stimulate cleavage of the single stranded regions of the hairpin loops of the substrates and hence made it more difficult to monitor resolution activity. The addition of diminazene aceturate, a viral topoisomerase inhibitor (Shaffer & Traktman, 1987) had no effect on the endonuclease activity (Fig. V-3, lane 12), although it significantly inhibited the relaxation of the supercoiled pUC plasmids in these extracts (data not shown). This was an unexpected observation considering that relaxed plasmids function as less efficient substrates for the *in vitro* resolution reaction (see below).

The resolution activity is sensitive to heat and to proteinase K but not to RNases. Two drugs tested that proved to be potent inhibitors of the resolving activity were actinomycin D (at 10 μg/ml) and ethidium bromide (at 10 μg/ml). We have not elucidated the mechanism of action for these inhibitors but they may act by altering the helical pitch of the target DNA and prevent the binding of critical proteins or by quantitatively intercalating into the substrate plasmids and forcing the resorption of extruded cruciforms.

Cleavage products that are consistent with a Holliday junction endonuclease. The circular substrate plasmid used in this assay contains a large inverted repeat of poxviral telomeric DNA sequences. Sufficient negative superhelical tension will cause the inverted repeat to be extruded from the plasmid to create a 4 armed DNA junction that mimics the structure of the Holliday junction intermediate obligatory to most models of homologous recombination (Dickie *et al.*, 1987, Merchlinsky *et al.*, 1988). Resolution of this Holliday junction analog in a sequence independent fashion can be accomplished by the nicking of opposing strands at the base of the junction with phage enzymes specific for branched DNA structures to yield a linear hairpin structure that is indistinguishable from the product of *in vivo* resolution (McFadden *et al.*, 1988). Nicking of the single stranded hairpins at the apex of the extruded cruciform can also result in the formation of discrete digestion products following cleavage with *Hind*III. The distinguishing feature between these two events is that the products formed by the nicking of hairpins will not be covalently linked, while the product of cleavage across the base of the cruciform will be a single self-complementary molecule. Thermal denaturation followed by quick chilling allows the two types of products to be distinguished because the self-complementary molecule with hairpin termini will rapidly reanneal and so will migrate as an undenatured full length fragment during gel electrophoresis. The product of hairpin nicking will fail to reanneal and its migration will be drastically altered during non-denaturing gel electrophoresis.

The fragments produced by treating pSCB1a with infected cell lysate followed by digestion with *Hind*III rapidly reanneal following boiling (Fig. V-4, lane 2) suggesting that at least some of the fragments produced are hairpin molecules. The hairpin fragments produced by the infected cell lysate are identical to the fragments created when pSCB1a that

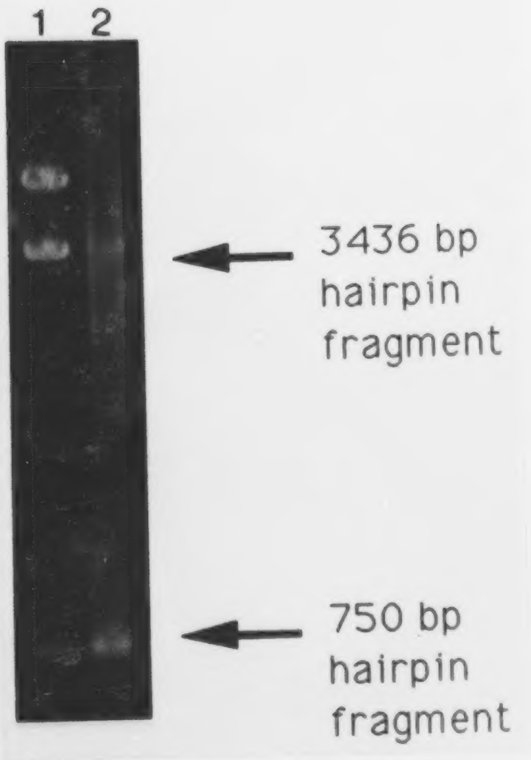


Figure V-4. Snap back analysis of the products generated from reaction of pSCB1a with the infected cell lysate. Following cleavage of the resolved plasmid with *Hind*III the reaction tubes were immersed in a boiling water bath for 5 minutes and then quick chilled by plunging the tubes into ice water. The DNA fragments were then fractionated on nondenaturing 0.7% agarose gels and visualized by ethidium bromide staining. Lane 1: undenatured DNA. Lane 2: DNA denatured and quick chilled. The 750 bp and 3436 bp snapback products are indicated by the arrows.

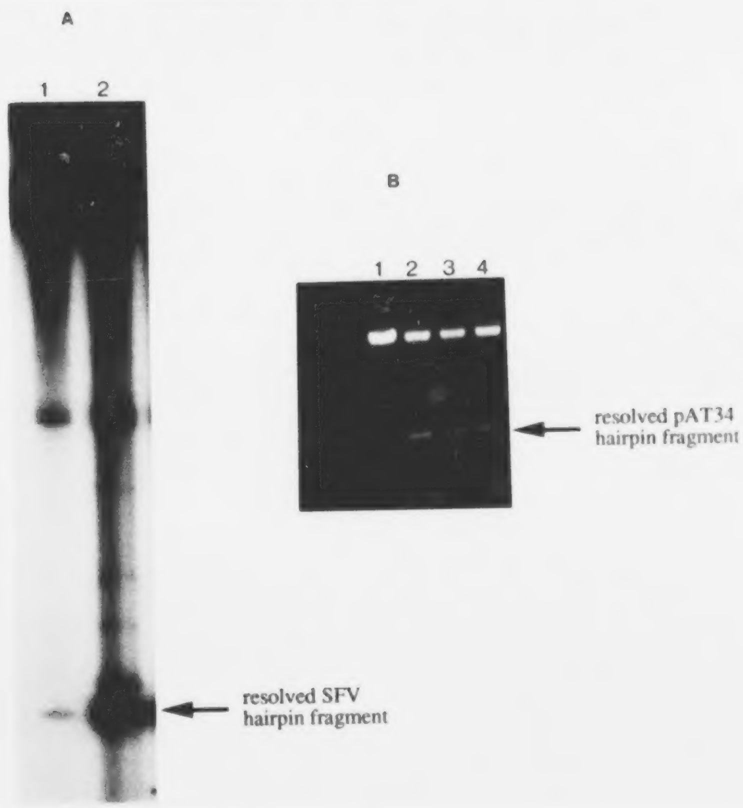
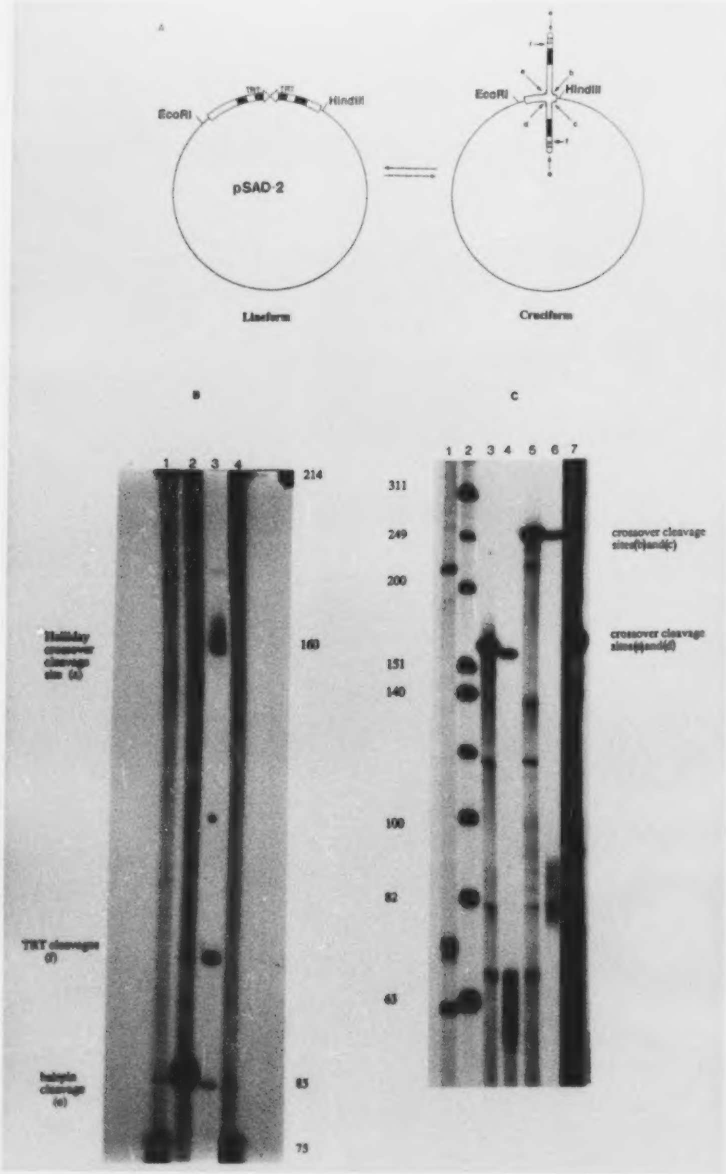

A
1
2
resolved SFV hairpin fragment
B
1
2
3
4
resolved pAT34 hairpin fragment

Figure V-5. (A) The plasmid pSAD-2 (DeLange & McFadden, 1987) carrying an inverted repeat insert of the core SFV TRT sequence was relaxed with topoisomerase and then treated with cell lysate followed by cleavage with *Hind*III (lane 1) or reacted with cell lysate and then *Hind*III without prior relaxation (lane 2). In each case the cleavage products were 3' end-labelled with $[\alpha\text{-}^{32}P]$ dATP and Klenow fragment. The end-labelled fragments were fractionated on an 8% nondenaturing polyacrylamide gel and visualized by autoradiography. The smaller pSAD-2 hairpin fragment generated by *Hind*III cleavage of the linearized plasmid is indicated with an arrow. (B) The plasmid pAT34 (Haniford & Pulleyblank, 1985) which contains a self-complementary d(AT)-d(AT) insert is resolved by the infected cell lysate. Lane 1: pAT34 linearized with *Bgl*II, lane 2, 3, and 4: pAT34 reacted with the infected-cell lysate at 30°C for 5, 10, and 20 mins and then cleaved with *Bgl*II. The hairpin fragment released by *Bgl*II cleavage is indicated by the arrow.

is extruding a cruciform is cleaved by the phage T7 gene 3 endonuclease (data not shown). Relaxation of plasmid DNA prior to treatment with an infected cell lysate makes most, but not all, of the DNA resistant to cleavage (Fig. V-5A, lane 1). This implies that the bulk of the resolution activity detected for this replicative intermediate structure may be targeted for the structure of the substrate DNA rather than on any specific sequence. By the same token, the residual level of resolution of completely relaxed plasmid substrates may well represent an independent activity that has more stringent sequence specificity (currently under study). We tested the infected cell lysates for sequence independent cleavage of branched DNA structures by utilizing the plasmid pAT34 which contains a self-complementary stretch of the sequence d(AT)-d(AT). This plasmid is known to readily extrude a cruciform that is susceptible to cleavage by T7 gene 3 endonuclease (Haniford & Pulleyblank, 1985). When tested with extracts from virus-infected cells the induced activity cleaved pAT34 in a manner similar to pSCB1a (Fig. V-5B, lanes 2, 3, and 4) indicating that the cleavages were not dependent upon the presence of any specific poxvirus sequences. A similar result was found when a non-mobile Holliday junction analog made of annealed oligonucleotides was used as the substrate (data not shown). Thus, unlike the sequence specific resolution of replicative intermediates observed *in vivo*, the major resolving activity detected in these lysates *in vitro* displays no sequence preference, consistent with a potential role in general recombination.

The major cleavage sites introduced into a cloned viral replicative intermediate were mapped on the plasmid pSAD-2,which contains an inverted repeat arrangement of the core TRT from SFV. The inverted repeats contained by plasmids such as pSAD-2 can exist in a lineform configuration or can be extruded into a cruciform (Fig. V-6A). Resolution of pSAD-2 can therefore occur by nicking opposing stands across the a-c or b-d axis at the base of the cruciform. The major product identified by denaturing gel electrophoresis of *in vitro* resolved pSAD-2 that had been reacted with the lysate and then cleaved with *Hind*III and 3' end-labelled was a set of fragments about 160 bases in length that were indicative of

Figure V-6. Positions of site specific nicks produced by the infected cell lysate on the cloned SFV telomere. Panel (A)The substrate plasmid pSAD-2 with the inverted repeat in the line form and in the cruciform configuration. Paired cleavages that would lead to resolution of the extruded cruciform are indicated by the arrows (a, b, c, d), nicking at the hairpin loops is indicated by the arrows (e) and nicks occurring within the TRT sequences are indicated by the arrows (f). Panel (B) lanes 1 and 4: pUC 19 digested with *Hinf*I size standards, the two fragments used for size determination are the 75 base fragment at the bottom of the gel and the 214 base fragment visible at the top of the gel. Lane 2: The substrate plasmid pSAD-2 cut with *Hind*III and *Afl*II to indicate the position of the 85 nucleotide band produced by creating nicks at the single stranded hairpin loop. Lane 3: pSAD-2 reacted with infected cell lysate, cleaved with *Hind*III and 3' end-labelled. The major cleavage site corresponding to the Holliday crossover cleavages at site (a) appears at 160 nucleotides from the labelled *Hind*III site and a possible TRT cleavage occurs at 95 nucleotides from the *Hind*III site. Cleavages at b, c and d are not detected by this protocol, but are detected in panel C. A minor amount of the labelled product mapping to the hairpin region may be the result of a low level of hairpin nicking but the specificity of this cleavage was not investigated. Panel (C) lane 1: pUC19 cleaved with *Hinf*I. Lane 2: ØX174 DNA cleaved with *Hinf*I for size standards. Lanes 3 and 4: pSAD-2 digested with infected cell lysate then cleaved with *Hind*III and finally 3' end-labelled (lane 3) to detect site a, or 5' end-labelled (lane 4) to detect site d. Lanes 5 and 6: pSAD-2 digested with infected cell lysate then *Eco*RI and finally 3' end-labelled (lane 5) to detect site c or 5' end-labelled (lane 6) to detect site b. Lane 7: pSAD-2 cut with phage T7 gene 3 endonuclease then *Hind*III and finally 3' end-labelled to detect site a. All samples were electrophoresed through a 0.4 mm 8% polyacrylamide gel containing 7 M urea and then visualized by autoradiography.

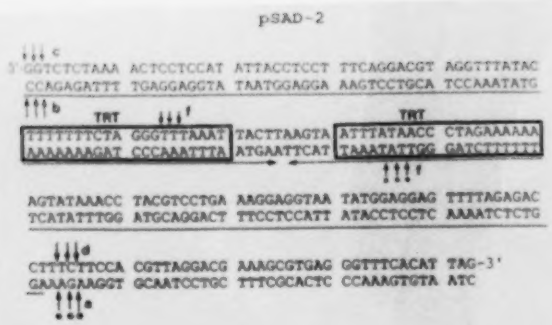

A
EcoRI
TRT
TRT
HindIII
pSAD-2
Lineform
a
b
c
d
e
f
EcoRI
HindIII
Cruciform
B
1
2
3
4
214
160
85
75
Holliday crossover cleavage site (a)
TRT cleavages (f)
hairpin cleavage (e)
C
1
2
3
4
5
6
7
311
249
200
151
140
100
82
63
crossover cleavage sites(b)and(c)
crossover cleavage sites(a)and(d)

substrate molecules that have been nicked across the a-c axis within the inverted repeat insert (Fig. V-6B, lane 3). In addition, specific nicking occurred in the region of the single stranded hairpin loop (site e) 85 bases from the labelled *Hind*III site; and internally at sequences corresponding to the TRT (site f). In addition to the 85 base size standard, the marker lane (Fig. V-6B, lane 2), shows a minor band at the position corresponding to a 95 nucleotide fragment. This appears to be a non-specific band or may be a site within the plasmid DNA that is particularly labile. pSAD-2 cleaved with the cytoplasmic lysate shows a doublet of bands at this position (Fig. V-6B, lane 3). This cleavage occurs within the TRT sequences but the significance of these nicks is not clear at this time. Nicks are introduced into pSAD-2 in a symmetric fashion on both the 3' and 5' strand of the inverted repeat insert at sites a, b, c, and d (Fig. V-6C), suggesting that the Holliday junction is resolved by nicking of the opposing strands across either the a-c or b-d axis at the base of the junction. Specifically, symmetrical cleavage of either axis is indicated by the formation of comparably sized fragments when either the 3' or the 5' strand of pSAD-2 is labelled after digestion with infected cell lysate followed by either *Hind*III (Fig. V-6C, lanes 3 and 4) or *Eco*RI (Fig. V-6C, lanes 5 and 6). These cleavage sites in pSAD-2 introduced by the poxviral induced endonuclease coincide precisely with the nicks made by the phage T7 gene 3 endonuclease (Fig. V-6C lane 7). The position of the faster migrating bands (Fig. V-6C, lanes 4 and 6) is not consistent with the initial nicks being introduced at the apex of the hairpin loop, rather the nicking appears to be located approximately 10 nucleotides 5' to the hairpin loop within the TRT region of the inverted repeat. The origin of this series of nicks is unclear: however, they may have been introduced by the labelling procedure (for instance, the labelling of internal nicks), since they are not reproduced when the cleaved DNA is labelled at the 3' end.

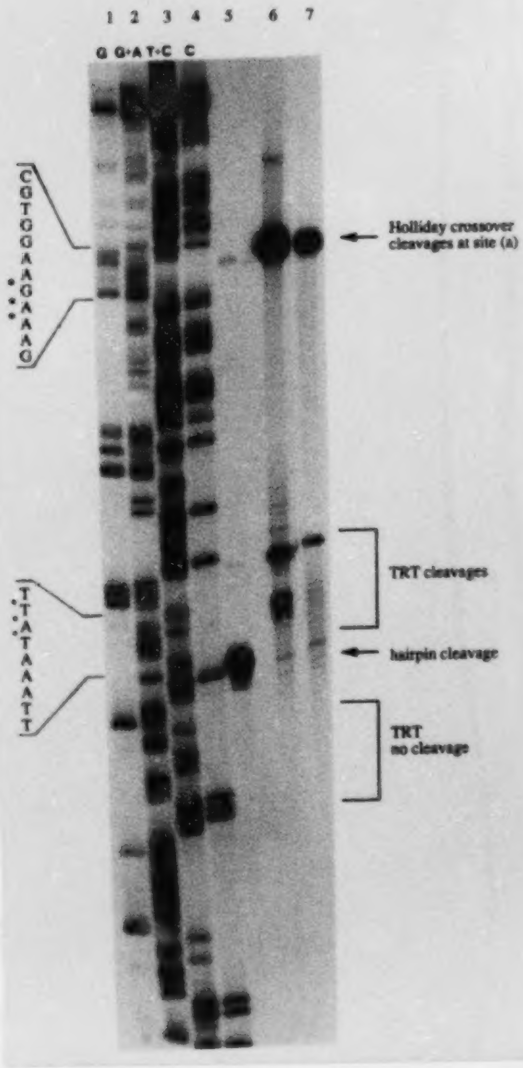
Mapping of in vitro cleavages within the viral TRT sequence. Fine mapping of the nicks induced in the plasmid pSAD-2 at sites most distal to the axis of symmetry indicate that cleavages occur directly at the point at which the two sides of the inverted repeat lose complementarity (Fig. V-7A). This cleavage also coincides with the nicks caused by the phage T7 endonuclease and illustrates the fact that branch migration to the end of these rather short inverted repeats only partially relaxes the negative supercoiling of the target plasmid and hence the crossover structures are relatively nonmobile at the most distal complementary nucleotides that permit free branch migration. Maxam & Gilbert sequencing illustrates that these cleavages are very precise and are not followed by random degradation (Fig V-7B).

Some faster migrating bands that are indicative of cleavages within the TRT sequence appear in Fig. V-7B lane 6. Importantly, these TRT cleavages are not symmetrical, as are those produced by the Holliday crossover resolving activity. Instead, as can be seen in lanes 6 and 7 in Fig. V-7B, when the lower strand (illustrated in Fig. V-7A) is probed by 3' end-labelling from the *Hind*III site, only the TRT copy distal to the dyad axis is cleaved, while the proximal TRT copy remains intact. When the upper strand is assessed, for example by 5' end-labelling from the *Hind*III site, the converse is observed (Fig V-6C lane 4), indicating that each copy of the TRT is cleaved only once on the strand 5' from the central axis of symmetry. Thus, these cleavages are highly specific for the viral TRT sequence and cleavage occurs in a fashion distinct from a standard Holliday crossover endonuclease. Another distinction can be made in the salt sensitivities of these two kinds of cleavages. As shown in lane 7 of Fig. V-7B, changing the salt conditions of the reaction buffer from 50 mM potassium glutamate to 100 mM KCl reduced the TRT cleavages while the Holliday crossover cleavages were unaffected. Thus, by these criteria the TRT cleavages behave as would be predicted for sequence-specific telomere resolution, while the Holliday crossover cleavages could also potentially reflect nonspecific recombination activities. The sites of the major cleavages introduced into the plasmids pSAD-2 and pSCB1a by this latter activity thus are structure specific rather than sequence specific, suggesting that it will resolve branched DNA structures and is probably not restricted to only viral telomere replicative intermediates. Plasmids identical to pSAD-2 but with single

Figure V-7. Fine mapping of nicks induced by the infected cell lysate on the substrate plasmid pSAD-2. Panel (A) the sequence of the pSAD-2 insert with the major Holliday-crossover cleavage sites a, b, c, and d, and the TRT cleavages at site f are denoted by vertical arrows. The axis of dyad symmetry in the insert is indicated by the underlining arrows and the TRT sequence is boxed. The vertical arrows at the outer extremities of the inverted repeat denote the symmetrical Holliday-crossover cleavages at site a, b, c, and d, and the asterisks indicate the site of the cleavages mapped in panel B. The vertical arrows within the TRT denote the strand-specific cleavages within the TRT and the asterisks indicate the cleavages that are mapped in panel B. Panel (B) Mapping of the cleavage sites induced by the infected cell lysate in pSAD-2 by Maxam and Gilbert sequencing. Lanes 1, 2, 3, and 4: pSAD-2 was cleaved with *Hind*III, then 3' end-labelled and cleaved with *Eco*RI. The labelled SFV fragment was isolated by nondenaturing polyacrylamide gel electrophoresis and subjected to Maxam and Gilbert sequencing reactions lane 1: G reaction, lane 2: G+A reaction, lane 3: T+C reaction, lane 4: C reaction. lane 5: pSAD-2 cleaved with *Hind*III and *Afl*II and then 3' end-labelled to mark the position corresponding to bands created by the introduction of nicks at the single stranded hairpin region. Lane 6: pSAD-2 digested with infected cell lysate in a reaction containing 50 mM potassium glutamate then *Hind*III and finally 3' end-labelled. The major cleavage product migrates as a 160 nucleotide fragment corresponding to cleavage at the sequences indicated by asterisks in panel (A). Some minor cleavage products can be observed migrating more rapidly through the gel. Lane 7: pSAD-2 treated identically to the sample in lane 6 however the resolution reaction was carried out in the presence of 100 mM KCl. The higher salt conditions decrease the spectrum of cleavages that are detected within the TRT sequences.


pSAD-2
c
5' GGTCTCTAAA ACTCCTCCAT ATTACCTCCT TTCAGGACGT AGGTTTATAC
CCAGAGATTT TGAGGAGGTA TAATGGAGGA AAGTCCTGCA TCCAAATATG
b
TRT
f
TRT
TTTTTTTCTA GGGTTTAAAT TACTTAAGTA ATTTATAACC CTAGAAAAAA
AAAAAAAGAT CCCAAATTTA ATGAATTCAT TAAATATTGG GATCTTTTTT
f
AGTATAAACC TACGTCCTGA AAGGAGGTAA TATGGAGGAG TTTTAGAGAC
TCATATTTGG ATGCAGGACT TTCCTCCATT ATACCTCCTC AAAATCTCTG
d
CTTTCTTCCA CGTTAGGACG AAAGCGTGAG GGTTTCACAT TAG-3'
GAAAGAAGGT GCAATCCTGC TTTCGCACTC CCAAAGTGTA ATC
e

B


1
2
3
4
5
6
7
G
G+A
T+C
C
C
G
T
G
G
A
A
G
A
A
A
G
T
T
A
T
A
A
A
T
T
Holliday crossover cleavages at site (a)
TRT cleavages
hairpin cleavage
TRT no cleavage

base mutations that abrogate *in vivo* resolution yet maintain the palindromic nature of the insert were tested in the assay and found to be converted to minichromosomes with comparable efficiency to that of pSAD-2 (data not shown) further indicating a lack of sequence specificity for the Holliday crossover endonuclease activity. We have also searched the newly available genomic sequence of vaccinia virus (Goebel *et al.*, 1990) for open reading frames that display similarity to the phage T4 and T7 resolving enzymes, but no significant homologies were identified.

D. Discussion

During the process of DNA replication the hairpin termini of poxviral genomes are transiently converted into palindromic "fusion" sequences that are the targets for virus specific resolution activities that convert the inverted repeat conformation into daughter hairpin termini (reviewed in DeLange & McFadden 1990). This resolution process can be monitored by the use of cloned poxvirus replicative intermediates that are replicated and resolved into linear hairpin-terminated minichromosomes when transfected into virus infected cells (DeLange *et al.*, 1986, Merchlinsky, 1990, Merchlinsky & Moss, 1989). This observation has allowed the delineation of the minimal target DNA sequences required for the resolution of poxvirus replicative intermediates *in vivo*. The complex process of telomere resolution is dependent upon the presence of two copies of a highly conserved twenty basepair sequence designated telomere resolution target (TRT) (DeLange & McFadden, 1987, Merchlinsky, 1990). Resolution of the telomere replicative intermediate also requires that the two copies of the TRT sequence be oriented such that they are inverted with respect to one another (Merchlinsky, 1990). The significance of inverted repeat nature of the replicative intermediate is clearly demonstrated by the observation that altering the orientation of the TRT sequences or inserting an asymmetric interruption between the TRT sequences abrogates or severely decreases the efficiency of the resolution process (McFadden *et al.*, 1988). Two models for the resolution of replicative intermediates have been put forth that are consistent with these data (DeLange & McFadden, 1990). One model involves site specific nicking and strand exchange within the TRT sequences followed by directed branch migration toward the axis of symmetry between the two TRT sequences. The second model invokes branch migration and the formation of a cruciform structure in the replicative intermediate. This secondary structure must be resolved by nicking the opposing strands of the Holliday junction at the base of the cruciform. The final products of either reaction are the same and both models mandate strand exchange coupled with branch migration to effect strand isomerization into daughter hairpin structures. However, the enzymes required to catalyze each reaction are different and the second model proposes that a transient cruciform structure would be an obligatory intermediate in the process.

In vitro the cloned replicative intermediate of the poxviral telomere is capable of extruding a cruciform structure that at its base is functionally analogous to a Holliday junction recombination intermediate and whose extruded arms are identical to the viral hairpin termini (DeLange *et al.*, 1986, Dickie *et al.*, 1987, Merchlinsky *et al.*, 1988). We have identified an activity present in poxvirus-infected cell extracts that shows a specificity for cleaving Holliday junctions in such extruded cruciforms. The presence of a virally induced Holliday junction endonuclease in infected cell lysates is not unexpected considering the tremendous amount of virus induced recombination that occurs *in vivo* (Ball, 1987, Evans *et al.*, 1988, Spyropoulos *et al.*, 1988). Most models of homologous recombination invoke the presence of such a crossover specific nuclease to resolve the Holliday junctions that result from strand exchange and branch migration. The activity that we have identified in this communication is able to resolve Holliday crossover junctions associated with cloned replicative intermediates into linear hairpin terminated minichromosomes that are indistinguishable from progeny molecules generated *in vivo* by replication and resolution. The "resolution" activity is virally induced, requires both viral DNA replication and the synthesis of late gene products but further analysis will be required to ascertain if it is required for both recombination and resolution or is restricted to just one process. Resolution *in vivo* requires viral late gene products but is not strictly dependent upon active DNA replication (Merchlinsky & Moss, 1989), while recombination can be detected at both early and late times in conjunction with DNA synthesis (Evans *et al.*, 1988). Results from lysates made from cells infected with the thermolabile vaccinia virus mutant ts53 at the non-permissive temperature and from cells infected with wild type virus in the presence of a DNA synthesis inhibitor such as ara C indicate that the resolving activity we have identified is not induced until the synthesis of late gene products begins. Proof that the activity is due to a viral gene product with late kinetics will require identification of the cognate gene and we cannot at this point exclude the possibility that the activity is expressed early but is only unmasked at late times in these extracts. The presence of a Holliday junction endonuclease in uninfected HeLa cells has been reported (Waldman & Liskay, 1988), however we have been unable to detect the cellular activity in the extracts prepared using this protocol either from uninfected cells or at early times after virus infection. The potential presence of both cellular and viral activities in the infected cell extracts would complicate the interpretation of some of the biochemical data, however it has been observed that in cells infected with vaccinia virus at high multiplicity most of the induced biochemical activities are directly attributable to viral gene products (Condit & Niles, 1990). Whether our inability to detect the cellular endonuclease is due to its lability or physical removal from these extracts is not known.

Although the activity specific for Holliday crossovers described here is consistent with a model of replicative intermediate resolution that calls for the extrusion of a cruciform from the inverted repeat there is no direct evidence for extruded poxviral cruciforms *in vivo*. Relaxed plasmid DNA substrates that do not possess extruded cruciforms are less efficiently resolved to minichromosomes than are supercoiled templates by these extracts but resolution can still be detected. This may indicate the presence of viral proteins that lead to unwinding of the inverted repeat TRT sequences and extrusion of a cruciform in these extracts or it may merely be an indication of how difficult it is to completely remove all cruciform structures from the substrate plasmids. Interestingly, the very active poxviral-encoded topoisomerase that is present within these extracts quickly relaxes all input plasmid DNAs and so we might expect to see some competition between the nicking and resolution of the plasmid cruciform structures and the relaxation of the substrate plasmids with concomitant resorption of the cruciform structures. However, inhibition of the viral topoisomerase with diminazene aceturate resulted in no detectable increase in the amount of resolved linear molecules (see Fig. V-3). *In vivo*, relaxed plasmids that are transfected into infected cells are replicated and resolved as efficiently as are transfected supercoiled molecules that extrude cruciforms (DeLange *et al.*, 1986). This suggests that cruciform extrusion mediated by superhelical tension alone is probably not an essential aspect of resolution of the inverted repeat structure *in vivo*. Indeed, it is predicted that the large linear replicated concatemeric DNA molecules that form the natural substrate for viral resolving activities would not be subjected to sufficient superhelical tension to cause quantitative extrusion of cruciforms. The possibility that viral proteins could serve to force the formation of an unusual secondary structure in the replicative intermediate remains a possibility. This is particularly germane in light of the finding that a single copy of the TRT sequence is capable of acting as a strong viral promoter at late times during infection (Stuart *et al.*, 1991). Melting and unwinding of promoter sequences by the viral transcriptional machinery (Vos *et al.*, 1991) could, in fact, serve to induce the formation of a secondary structure such as a cruciform that would be cleavable by the branched structure specific nuclease described here.

The resolution process *in vivo* is strictly dependent upon the sequence and orientation of the TRTs in the replicative intermediate (DeLange & McFadden, 1990). Strand-exchange between the arms of the inverted repeat and branch migration between the two TRT sequences are required for resolution of the replicative intermediates (Merchlinsky, 1990), and in fact is the only way to explain the regeneration of the "flip" and "flop" pattern of hairpin termini that are observed in all poxviruses that have been examined (DeLange *et al.*, 1986). In order to explain the segregation of daughter hairpin structures from a precursor inverted repeat substrate, transient cleavage must occur at or near the TRT sequences at some time during the resolution process *in vivo*. Thus, the sequence specific cleavages that we observed within the TRT sequences by these virus-infected extracts are particularly significant and suggest that at least some aspects of the resolution mechanism *in vivo* have been maintained. At the present time we are unable to deduce whether the Holliday crossover endonuclease is linked with the TRT cleaving activity, either physically in a complex or functionally in a common resolution pathway. One possibility is that the role of the Holliday junction endonuclease activity in virus replication is not only the sequence specific resolution of replicative intermediates but also the resolution of any branched junctions that are incurred during the replication and recombination of viral DNA. Further analysis, especially the generation of mutants in the cognate viral gene(s), will be required to establish the precise role of these two activities in the viral replicative cycle.

In several significant ways the poxvirus replicative cycle is more like that of the bacteriophages T4 and T7 than that of nuclear mammalian viruses. Convoluted interactions occur amongst the pathways for phage T4 transcription, replication, recombination and packaging such that they utilize many common enzymes and attempts to uncouple these processes with mutational blocks or inhibitors often result in pleiotropic effects on DNA metabolism (Mosig *et al.*, 1984). Strand invasion and recombination in phage T4 has been demonstrated to result in the formation of primers for DNA synthesis and mutants that are deficient in strand invasion demonstrate an arrest of DNA replication following the early rounds of synthesis (Cunningham & Berger, 1977). Recombination can occur in the absence of replication, however it occurs with much less frequency than when DNA replication is allowed to proceed (Mosig, 1983). The phage T4 gene 49, which encodes a Holliday junction resolving enzyme (Jensch & Kemper, 1986, Mizuuchi *et al.*, 1982), is expressed at late times (Frankel *et al.*, 1971) and is required to trim the branched structures that comprise the nascent genomic DNA and cleave the concatemeric structures into single unit length genomes in the packaging reaction (Jensch & Kemper, 1986). Bacteriophage T7 also encodes a Holliday junction cleaving enzyme that cleaves concatemeric DNA in a packaging reaction (deMassey *et al.*, 1987). DNA replication and recombination occur concurrently in poxvirus-infected cells and to date attempts to separate the two processes with conditional lethal mutants (Merchlinsky, 1989) or inhibitor drugs (Evans *et al.*, 1988) have been unsuccessful. Efficient recombination in poxviruses is dependent upon DNA synthesis but there is no obvious requirement for late gene expression *per se* which usually commences after DNA replication is initiated (Merchlinsky, 1989). This is in contrast to the resolution of the replicative intermediates which occurs only under conditions that are permissive for late gene expression (Merchlinsky & Moss, 1989). No poxvirus mutants have yet been identified that are solely deficient in recombination or resolution and all temperature sensitive viruses that have been found to be defective in recombination are also deficient in DNA synthesis (Merchlinsky, 1989). Most of the viral mutants identified that are defective in replicative intermediate resolution are also deficient in the expression of late genes or the stability of late mRNA (Merchlinsky & Moss, 1989). The single exception is the vaccinia virus mutant ts9383 which is defective in telomere resolution due to a mutation in the small subunit of the mRNA capping enzyme (Carpenter & DeLange, 1991).

The pleiotropic nature of the defects in viral recombination and replicative intermediate resolution are similar to the wide ranging defects in DNA metabolism displayed by phage mutants that are defective in recombination or replication proteins (Mosig, 1983). During the phage T4 replicative cycle it has been shown that both replication and recombination are carried out by multisubunit enzymes or protein "machines" (Alberts, 1984). Many of the proteins that reside within these complexes are utilized for both replication and recombination activities. The multicomponent nature of these complexes means that the constituent enzymes must associate with low affinity in order to allow a mixing and matching of activities between different complexes in different places. This intrinsic linkage of activities and efficient use of enzymes results in functional complexes that are inherently unstable and refractory to purification. This situation may also be the case for sequence specific resolution of poxviral replicative intermediates. For example, the poxviral transcription complex utilizes different subunits at early and late times during infection (Thompson *et al.*, 1989). The transcriptional assembly includes a variety of subunits including the viral capping enzyme (Broyles & Moss, 1987) and possibly a host RNA polymerase II subunit (Moyer, 1987), and thus recruitment of the multisubunit transcription complex to the TRT may target this sequence to a resolution or strand exchange factor that is associated with the transcription complex. The activity that we have identified may be one part of that complex but it is likely that more factors remain to be identified before *bona fide* resolution can be accurately reconstituted *in vitro*.

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Chapter VI

High levels of Genetic Recombination Among Cotransfected Plasmid DNAs in Poxvirus-Infected Mammalian Cells

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A. Introduction

General recombination, the exchange of genetic information by breakage and crosswise reunion of DNA strands between homologous sequences, is a fundamental event in the generation of genetic diversity. Homologous recombination is common not only to eukaryotic and prokaryotic cells but also to a variety of prokaryotic phage, including; T4 (Hamlet & Berger, 1975, Mosig, 1987), T7 (Studier, 1969), and λ (Smith, 1983) and to eukaryotic viruses, SV40 (Wake & Wilson, 1980), adenovirus (Volkert & Young, 1983), and HSV (Delius & Clements, 1976). In the cases of phage T4 and T7 it is known that homologous recombination is required not so much for the generation of genetic diversity but because it serves a critical function in the priming of DNA replication (Formosa & Alberts, 1986, Studier, 1969). Due to their dependence upon the process of recombination both of these phage encode some of the enzymes that are required in the homologous recombination pathway (Kerr & Sadowski, 1975, Mizuuchi *et al.*, 1982). Eukaryotic nuclear viruses are also subjected to high levels of recombination. The genetic maps of these viruses that have proven to be colinear with the physical map of the genomes (Volkert & Young, 1983). Recombination of HSV DNA occurs with very high efficiency and this has allowed the genome to be manipulated *in vivo* by marker rescue and gene replacement techniques (Post & Roizman, 1981). The genomes of these eukaryotic viruses reside within the host cell nucleus and it seems likely that the viral DNA is subjected to recombination by the cellular activities which have been shown to catalyze both homologous and illegitimate recombination of exogenously added DNA (Razzaque *et al.*, 1983, Roth & Wilson, 1985). In the case of a very large and complex virus such as HSV it is not clear which, if any viral enzymes are specifically involved in the recombination of viral DNA.

Not all eukaryotic viruses replicate within the host cell nucleus with the opportunity to utilize the cellular recombination apparatus. Members of the poxvirus family of eukaryotic viruses replicate exclusively within the cytoplasm of the infected cell with little or no involvement of a host nucleus (Hruby *et al.*, 1979). While the cytoplasmic location of DNA synthesis confers autonomous replication to the poxviruses it also means that all of the replicative functions must be encoded within the viral genome. During the replicative cycle poxvirus DNA is subjected to high levels of homologous recombination. This was initially shown by coinfecting cells with two distinct viruses and then demonstrating that plaques formed by the progeny expressed a combination of the genetic markers (Fenner & Comben, 1958). This high frequency recombination was further demonstrated by the creation of a linkage map of the rabbitpox virus genome based on pock morphology (Gemmel & Cairns, 1959). The homologous recombination induced in poxvirus infected cell cytoplasms is not unique to viral DNA. It has been shown that exogenously added DNA that has sequence homology to poxvirus DNA will recombine with the replicating viral DNA. This process forms the basis of marker rescue (Nakano *et al.*, 1982), and allows the mapping of viral genes (Weir *et al.*, 1982), the introduction of foreign genes into the viral DNA, and the replacement or disruption of viral genes. In spite of the utility of poxvirus recombination for the study of viral genes and the generation of recombinant viral vaccine vectors, the mechanism of homologous recombination in poxvirus infected cells is poorly understood. It appears that both intramolecular and intermolecular recombination take place within the viral DNA (Ball, 1987) as well as within and between exogenous plasmid DNA that has been introduced into the poxvirus infected cells (Merchlinsky, 1989). Poxvirus replicative enzymes display a high degree of promiscuity. Any circular plasmid DNA that is introduced into poxvirus infected cells is replicated in a sequence independent fashion into a high molecular weight head-to-tail tandem array of plasmid DNA (DeLange & McFadden, 1986). This observation can be explained by a rolling circle mode of DNA replication or by a mechanism where extensive genetic recombination in conjunction with DNA replication results in the generation of tandem arrays of plasmid DNA in the infected cell cytoplasm.

In this report we demonstrate that when poxvirus infected cells are cotransfected with two different but homologous plasmids, very high levels of intermolecular and intramolecular recombination are observed. This recombination is dependent upon the presence of the infecting poxvirus. No conditional lethal viral mutants have been identified that are defective exclusively in recombination (Merchlinsky, 1989). However, we feel that these studies implicate virally encoded gene products as being responsible for the expanded recombination potential in infected cells rather than an induced activity of cellular enzymes.

B. Materials and methods

Strains and viral stocks. Plasmids were prepared from Escherichia coli (E. coli) strains provided by R. Kolodner. These were RDK1233 (pRDK41), RDK1388 (pRDK35), and RDK1389 (pRDK39), all of which are ampicillin-resistant transformants of JC10287[Δ(srlR-recA)304 thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 str-31 tsx-33 supE44] (Doherty *et al.*, 1983). In transformation assays (Symington *et al.*, 1983) E. coli RDK1400 (recA13 thr leuB6 thi thyA trpC1117 hsdR12 hsdM12 Str^r) was used. Vaccinia virus IHD-W (provided by S. Dales) was propagated by infection of BGMK monkey cells. Shope fibroma virus (SFV) (Kasza) and myxoma virus (Lausanne) were propagated by low multiplicity infection of monolayer cultures of SIRC rabbit cells. Both cell lines were from the American Type Culture Collection (ATCC). Viral stocks were prepared and purified as described previously (Wills *et al.*, 1983).

Enzymes, chemicals, and media. Restriction enzymes were purchased from Bethesda Research Laboratories, Boehringer Mannheim Biochemicals, or New England Biolabs, and were used as directed by the manufacturers. E. coli DNA polymerase I was a gift from Dr. A. R. Morgan and was purified by the procedure of Jovin *et al.*, (1969). T4 DNA ligase was a gift from R. Kolodner and was purified by a modification of the procedure of Panet *et al.*, (1973). Proteinase K was purchased from Sigma Chemical Co. Bacterial growth media were from Difco Laboratories. BGMK and SIRC cells were propagated as monolayer cultures in Dulbeco's modified Eagle's medium (Gibco Laboratories) supplemented with 5% fetal calf serum (Flow Laboratories). Phosphonoacetic acid (PAA), cytosine-β-D-arabinofuranoside (ara C), and actinomycin D were purchased from Sigma Chemical Co.

Nucleic acids. Plasmid DNA was prepared from cells grown to stationary phase overnight in Fraser's medium supplemented with 50 μg of thymine per ml and 180 μg of ampicillin per ml. Plasmids were isolated by a large scale alkaline lysis procedure (Birnboim & Doly, 1979) and the supercoiled DNA was purified by using two successive ethidium bromide-CsCl gradients.

Transfection of virus infected cells. Monolayers containing $2x10^6$ to $3x10^6$ SIRC cells were infected with the specified poxvirus at a multiplicity of infection (MOI) of 1 infectious unit per cell and then transfected 1 hr (vaccinia virus) or 3 hrs (myxoma virus and SFV) later by the addition of 50 ng of $CaPO_4$-precipitated plasmid DNA. Unadsorbed DNA and virus were removed by washing the monolayer first with phosphate buffered saline (PBS; 145 mM NaCl, 4 mM KCl, 10 mM phosphate pH 7.2) plus 5 mM EDTA and then with PBS 3 hrs after the addition of DNA, and fresh medium was then added. When plasmid mixtures were applied, they were first coprecipitated and then 50 ng of total DNA was added per $2x10^6$ to $3x10^6$ cells. As described previously, no carrier DNA is required for the efficient uptake of $CaPO_4$ precipitated plasmid DNA into the cytoplasm of poxvirus-infected cells (DeLange & McFadden, 1986). After the incubation times indicated, total cellular DNA was recovered from infected/transfected cells by lysing the monolayer on the plate with 300 μl of 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.5% SDS. The cell lysate was then incubated for 4 hrs at 37°C in the presence of 2 mg per ml proteinase K. The DNA was then purified by extraction with equal volumes of; phenol, phenol:chloroform (1:1) and chloroform followed by precipitation with ethanol. Prior to further analysis the DNA was resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA and then treated with

RNase A (final concentration 100 μg/ml) for 30 min at 37°C. The concentration of each DNA sample was determined by fluorimetric measurement (Morgan *et al.*, 1979). All of the times given in the figure legends are times post-transfection.

Analysis of recombinant products by Southern blotting. Total cellular DNA harvested from infected/transfected cells was digested with restriction enzymes as indicated in the figure legends. 500 ng of each DNA was applied per well and electrophoresed through 0.7% agarose gels. The DNA was transferred to nitrocellulose by a capillary transfer procedure (Southern, 1975), and then fixed to the membrane by baking at 80°C for 2 hrs. The blots were then prehybridized in a solution of 50% deionized formamide, 3 x SSC (450 mM NaCl, 45 mM sodium citrate pH 7.2), 1 x Denhardt's solution, 20 μg/ml denatured calf thymus DNA, 200 μg/ml yeast tRNA and probed with nick translated [^{32}P] labelled pBR322 and λ DNA (specific activity, approximately 10^8 cpm/μg), and the blots were visualized by exposure to Kodak AR film with Cronex intensifier screens. Bacteriophage λ DNA digested with *Eco*RI and *Hind*III served as a molecular weight marker. Complete restriction enzyme digests were assured by examining the ethidium bromide stained agarose gels prior to Southern blotting. Viral DNA restriction fragments were visible superimposed upon the heterogeneous smear of cellular DNA digestion products (see Fig. VI-3 and VI-4).

A determination of the frequency of plasmid recombination was carried out by Dr. David Evans using two procedures: (i) The autoradiographs generated from the Southern blots of the *Xho*I digested DNA recovered from poxvirus infected cells were scanned with an LKB Ultroscan XL Laser densitometer. Integration was performed either by the cut-and-weigh method or by using integration values calculated by the densitometer. The percent recombination was calculated using the following formula:

$$\%\ \text{recombination} = \frac{\Sigma\ (\text{integrated area of recombinant restriction fragments})}{\Sigma\ (\text{integrated area of parental and recombinant fragments})} \times 100$$

(ii) A portion of the DNA recovered from transfected cells was digested with *Eco*RI, repurified by phenol extraction in the presence of 100 μg of tRNA per ml, ethanol precipitated, and circularized at a DNA concentration where $j = 10i$ with T4 DNA ligase (Maniatis *et al.*, 1982). After purification by phenol extraction and ethanol precipitation, the DNA was transformed into competent E. coli (Morrison, 1979) and plated on LB plates containing 100 μg of ampicillin per ml. Transformants were replica plated onto LB plates containing 100 μg of ampicillin per ml and 20 μg of tetracycline per ml to determine the recombination frequency. The recombination frequency was calculated from the following formula:

$$\% \text{ recombination} = \frac{(\text{ampicillin}^r + \text{tetracycline}^r \text{ colonies})}{(\text{ampicillin}^r \text{ colonies})} \times 100$$

C. Results

Experimental design. The plasmids used for this study are illustrated in Fig. VI-1. Plasmids pRDK35 and pRDK39 contain *Xho*I linkers inserted into *Taq*I sites that disrupt and inactivate the tetracycline resistance gene of pBR322 (Doherty *et al.*, 1983). The plasmid pRDK41 is a circular dimer containing pRDK35 joined in tandem to pRDK39 and was used to monitor intramolecular recombination events (Doherty *et al.*, 1983). Genetic recombination occurring within the tetracycline resistance genes of these plasmids can be detected by selection for the rescue of a functional tetracycline resistance gene or by restriction mapping (Doherty, *et al.*, 1983, Symington *et al.*, 1983). The use of restriction mapping to detect a simple single crossover event between the monomeric constructs pRDK35 and pRDK39 is illustrated in Fig. VI-2. Such an event occurring within the tetracycline resistance gene would generate (after digestion with *Xho*I) diagnostic restriction fragments of 1.24 and 7.48 kilobases (kb). Gene conversions or multiple rounds

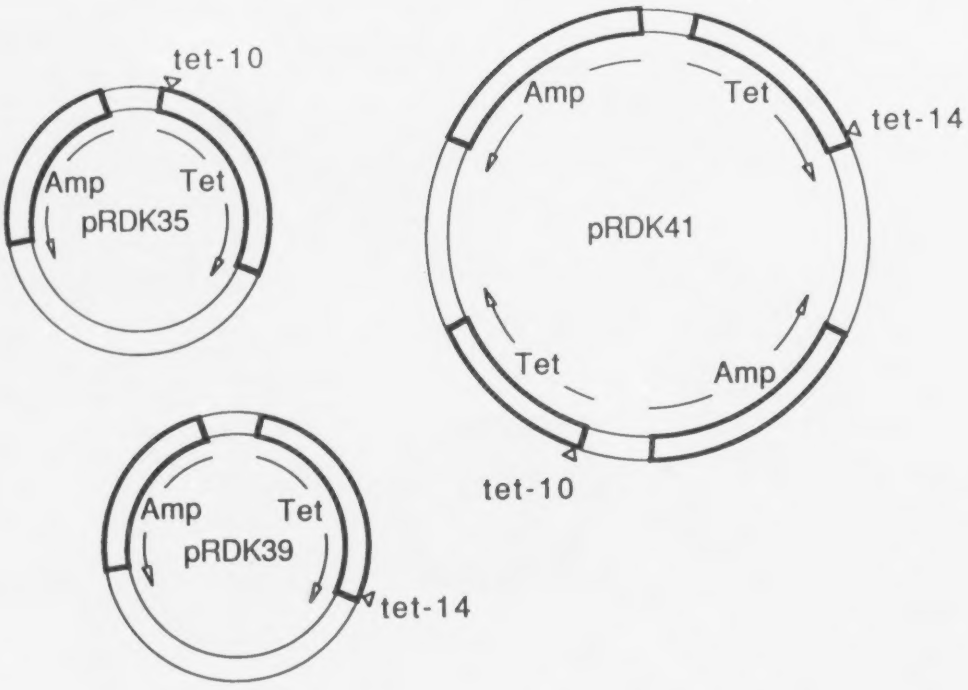

tet-10
Amp
Tet
pRDK35
Amp
Tet
tet-14
pRDK41
Tet
Amp
tet-10
Amp
Tet
pRDK39
tet-14

Figure VI-1. The structure of plasmids used to quantitate recombination in transfected cells. The three plasmids are derivatives of pBR322, and their construction has been described (Doherty *et al.*, 1983).The *tet*-10 and *tet*-14 alleles are *Xho*I linker insertions at *Taq*I sites that inactivate the tetracycline resistance gene. The two loci are separated by 1.24 kb.

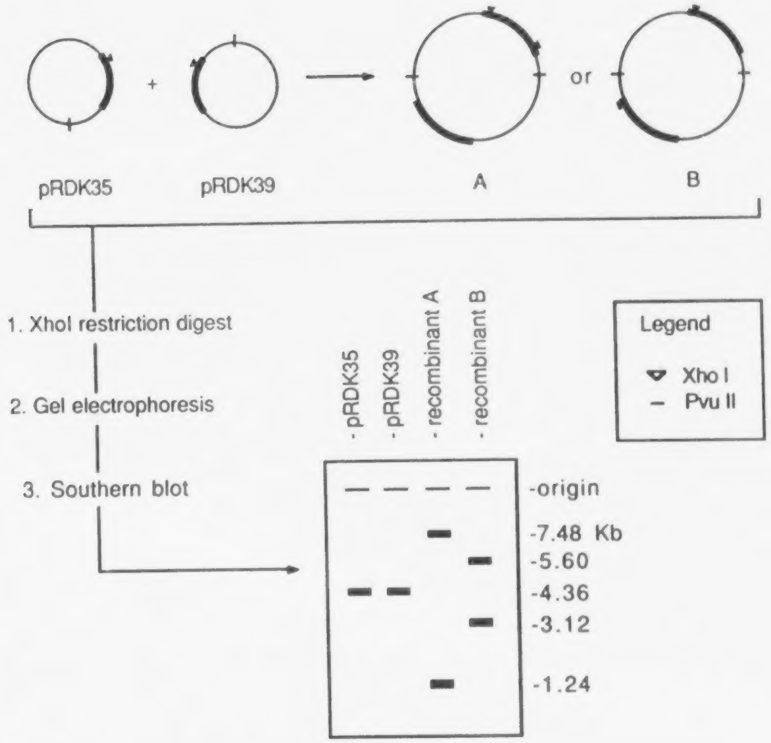

pRDK35
pRDK39
A
or
B
1. XhoI restriction digest
2. Gel electrophoresis
3. Southern blot
- pRDK35
- pRDK39
- recombinant A
- recombinant B
-origin
-7.48 Kb
-5.60
-4.36
-3.12
-1.24
Legend
Xho I
Pvu II

Figure VI-2. The protocol used to quantitate recombination by Southern blotting. Only the results of initial, single intermolecular recombination events are shown. A single reciprocal intermolecular recombination event between pRDK35 and pRDK39 can generate a dimeric product. The formation of these molecules can be detected by digestion with *Xho*I, agarose gel electrophoresis, and Southern blotting with a ^{32}P-labelled vector probe. New restriction fragments 7.48 and 1.24 kb in length are expected products of a reciprocal event occurring within the tetracycline resistance gene, whereas 3.12 and 5.60 kb fragments are characteristic of events occurring outside of the tetracycline gene. Further rounds of reciprocal and /or nonreciprocal recombination would also generate these species, plus other characteristic higher order restriction fragments.

of recombination would generate these and other, predictable patterns of recombinant restriction fragments that can be readily detected by Southern blotting.

Recombination between transfecting plasmids is dependent upon poxvirus infection. In preliminary experiments monolayers of SIRC cells were infected with SFV or myxoma virus at a multiplicity of 1 focus forming unit (ffu) per cell or with vaccinia virus at a multiplicity of 1 plaque forming unit (pfu) per cell. The infected cells were then transfected with plasmid DNA, and after 24 hrs after which the total cellular DNA was recovered. It has been shown previously that under these conditions virtually any circular input plasmid DNA will be replicated in a sequence independent fashion into high molecular weight concatemeric arrays by viral *trans*-acting factors (DeLange & McFadden, 1986). After digestion with *Xho*I the DNA fragments were fractionated by agarose gel electrophoresis and Southern blots of these gels were probed with [^{32}P]-labelled pBR322 DNA (vector sequences). Recombinant products were readily recovered from poxvirus-infected cells 24 hrs after transfection (after replication of the endogenous viral genomes was essentially completed) but not 3 hrs after transfection (prior to the initiation of viral DNA replication) or at any time in uninfected cells (Fig. VI-3 and VI-4). This is most readily apparent in the laddered array of recombinant fragments observed, for example in myxoma virus- and SFV-infected cells transfected with a mixture of pRDK35 and pRDK39 or with pRDK41 alone (Fig. VI-3B, lanes 16 and 17; Fig. VI-4B, lanes 7 and 8). This distribution of new fragment sizes can be explained by the formation of head-to-tail repeats of plasmid DNA flanked by multiple orientations of the *tet-10* and/or *tet-14 Xho*I linker insertion sites. Both intermolecular and intramolecular events occurred with approximately equal frequency, since ladders of similar distribution and intensity were observed in cells transfected with a mixture of pRDK35 and pRDK39 (Fig. VI-3B, lane 16) or with the dimeric pRDK41(Fig. VI-3B, lane 17). The generation of intermolecular recombinants is clearly dependent upon the presence of both pRDK35 and pRDK39, because when cells were transfected with either plasmid alone, only 4.36-kb fragments of parental

Figure VI-3. Recombination between transfected plasmid DNA in SFV infected SIRC cells. Monolayers of SIRC cells were infected with SFV or mock infected and transfected with the DNA indicated below. DNA was recovered at 3 hr or at 24 hr post-transfection and digested with *Xho*I, electrophoresed through 0.7% agarose gels, stained with ethidium bromide (A), and then blotted and hybridized with ^{32}P-labelled pBR322 ands exposed for 15 hr to detect recombinants. (B) Size standards (lane 9) are derived from λ *Eco*RI-*Hind*III digests. Harvest times (hrs) and infection condition are indicated between the two panels. Transfected DNAs were pRDK35 (lanes; 1, 5, 10, and 14), and pRDK39 (lanes; 2, 6, 11, and 15), pRDK35 plus pRDK39 (lanes; 3, 7, 12, and 16) and pRDK41 (lanes; 4, 8, 13, and 17). (C) Seven-day exposure of lanes 5 through 8 to illustrate that the level of recombination in uninfected cells is low under these conditions. Note that pRDK41 is one of the recombinants that can be formed between pRDK35 and pRDK39. The 3.12 and 5.60 kb restriction fragments characteristic of this event (lane 16) and of unrecombined pRDK41 (lanes 4 and 13) are indicated by arrows.

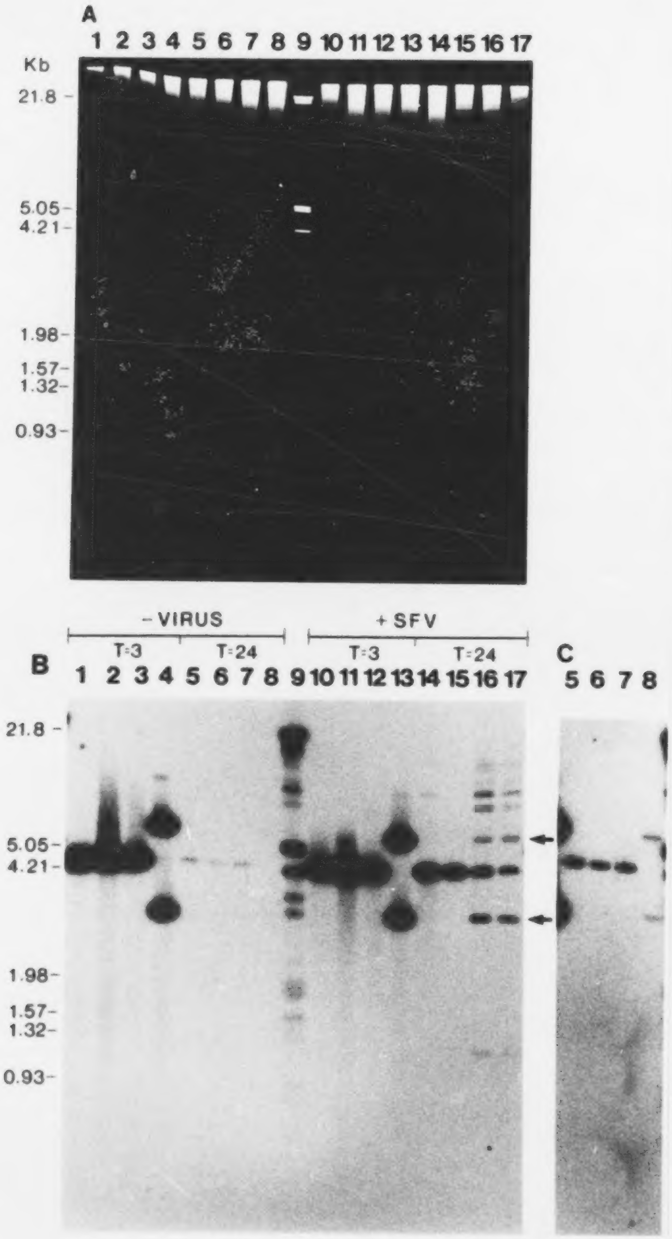
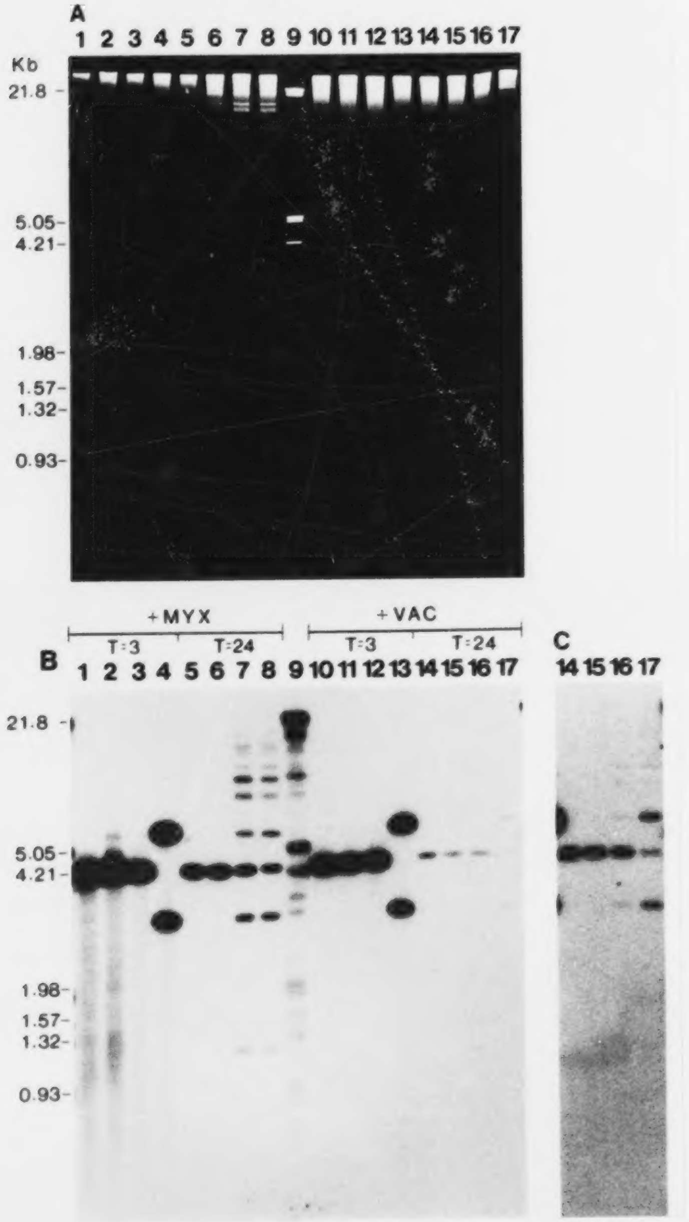

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Kb
21.8
5.05
4.21
1.98
1.57
1.32
0.93
- VIRUS
+ SFV
T=3
T=24
T=3
T=24
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
21.8
5.05
4.21
1.98
1.57
1.32
0.93
C
5 6 7 8

Figure VI-4. Recombination between transfecting plasmid DNAs in myxoma virus and vaccinia virus-infected SIRC cells. The experiments were performed as indicated in Fig. VI-3, and the results are presented in an identical manner. DNAs are pRDK35 (lanes; 1, 5, 10, and 14), pRDK39 (lanes; 2, 6, 11, and 15), pRDK35 plus pRDK39 (lanes 3, 7, 12, and 16), and pRDK41 (lanes; 4, 8, 13, and 17). (C) Seven-day exposure of lanes 14 through 17 to better illustrate the detectable levels of recombination in vaccinia virus infected cells.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Kb
21.8
5.05
4.21
1.98
1.57
1.32
0.93
+MYX
+VAC
T:3
T:24
T:3
T:24
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
21.8
5.05
4.21
1.98
1.57
1.32
0.93
C
14 15 16 17

configuration could be recovered 24 hrs post-transfection (e.g., Fig. VI-3B, lanes 14 and 15).

The observed recombination was dependent upon poxvirus infection in two ways. First as shown below, recombinant fragments were derived from newly replicated DNA, and this replication was necessary to maintain and amplify the plasmid copy number. In the absence of infection only a small fraction of the input plasmid sequences survived for 24 hrs in culture (Fig. VI-3B, lanes 5 through 8). Although at 3 hrs post-transfection both infected and uninfected cells appeared to have taken up equal amounts of plasmid DNA (Fig. VI-3B, compare lanes 1 through 4 with lanes 10 through 13). It was also apparent that a detectable level of recombination occurred only in the poxvirus-infected cells (Fig. VI-3B compare lanes 7 and 8 with lanes 16 and 17). Although some of the input plasmid DNA survived for 24 hrs in uninfected cells, overexposed Southern blots showed that this DNA still retained only a parental configuration of the *XhoI* restriction sites (Fig. VI-3C). Note that this observation does not imply that uninfected SIRC cells are incapable of homologous recombination, but rather that within the 24 hr period examined, recombination frequencies in uninfected cells were below levels detectable by the methods described here. The absence of carrier DNA in these transfections and the small amount of input plasmid DNA may both have contributed to the apparently low levels of recombination in the uninfected SIRC cells. Furthermore, the observed amplification of transfected plasmid DNA is believed to occur in the cytoplasm of the poxvirus-infected host cells (DeLange & McFadden, 1986), a site that may be devoid of detectable cellular recombination enzyme activities in the uninfected controls.

The three poxviruses were not equally proficient in the production of recombinant molecules. With our transfection protocol, SFV (Fig. VI-3B, lanes 16 and 17) and myxoma virus (Fig. VI-4B lanes, 7 and 8) clearly catalyzed higher levels of recombination than did vaccinia virus (Fig. VI-4B, lanes 16 and 17). Densitometric analysis showed that the fraction of DNA present as recombinant fragments 24 hrs after transfection of SFV- and myxoma virus-infected cells was 57 to 63% and 61 to 66%, respectively. A similar analysis involving an overexposure of the same southern blots showed that at most only 25 to 36% of the DNA recovered from vaccinia virus-infected cells was recombinant (Fig. VI-4C, lanes 16 and 17 respectively). Cells were shown to be productively infected to a comparable degree in each case, since similar amounts of virus-derived *Xho*I fragments were readily detected by ethidium bromide staining prior to Southern transfer (Fig. VI-3A and VI-4A). This lower degree of recombinational activity in vaccinia virus infected cells cannot therefore simply be explained by the failure of vaccinia virus to replicate in the host SIRC cells.

Timing of plasmid recombination. The kinetics of recombination in SFV-infected cells transfected with a mixture of pRDK35 and pRDK39 or with pRDK41 alone were further examined. DNA was recovered at various times after transfection, and recombination was assayed by *Xho*I digestion as described above. Essentially identical results were obtained by using a mixture of the monomeric plasmids or pRDK41 alone, so only the results obtained from the transfection with pRDK41 are shown (Fig. VI-5).

Recombinant plasmid DNA was first detected about 6 to 8 hours post-transfection, and a limit pattern was reached by approximately 12 hrs (Fig. VI-5, lanes 3 through 5). The first recombinants detected were the products predicted for single recombination events (for example, the 4.36 kb monomer product). Subsequent recombination events generated higher molecular weight tandem repeats of pBR322 flanked by the *tet*-10 and/or *tet*-14 *Xho*I linker insertion sites. Whether recombination events terminated 10 to 12 hrs after transfection could not be determined by this protocol because further rounds of recombination would not be expected to appreciably alter these profiles (Fig. VI-5, lanes 5 through 8). Tandem repeats in excess of 13 to 15 kb were observed, although this may have reflected an equilibrium limit rather than the actual termination of recombination events *per se*.

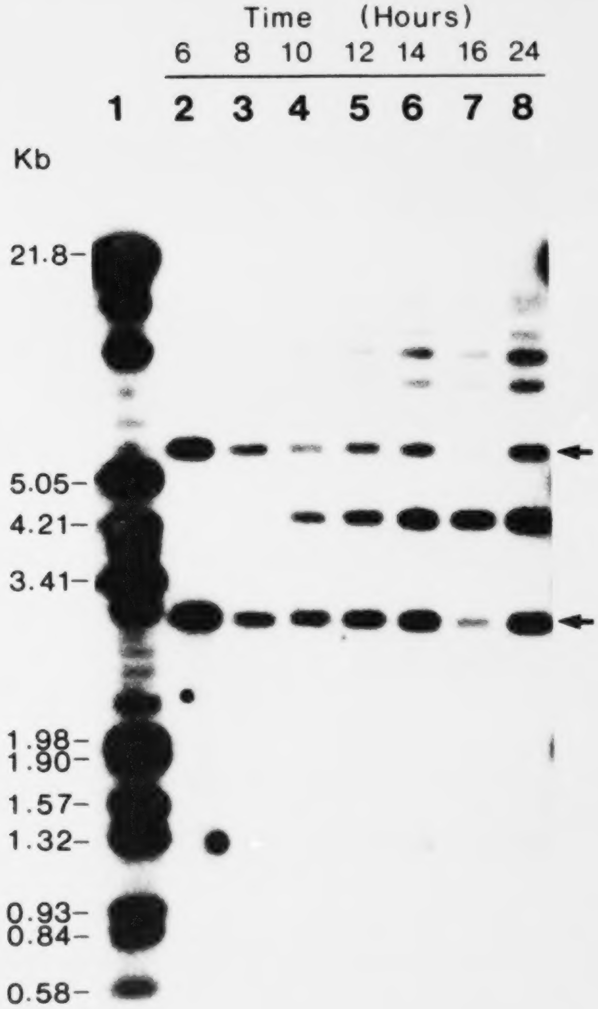

Time (Hours)
6 8 10 12 14 16 24
1 2 3 4 5 6 7 8
Kb
21.8–
5.05–
4.21–
3.41–
1.98–
1.90–
1.57–
1.32–
0.93–
0.84–
0.58–

Figure VI-5. Kinetics of recombination following transfection of pRDK41 DNA into SFV-infected SIRC cells. Monolayers of SIRC cells were infected with SFV and transfected with pRDK41, and the DNA was recovered at the times indicated (lanes; 2 through 8). DNA was digested with *Xho*I, electrophoresed and blotted. λ DNA cleaved with *Eco*RI and *Hind*III was used as a size marker. Arrows indicate the two parental pRDK41-derived *Xho*I fragments of 3.12 and 5.60 kb.

Properties of newly recombined DNA. The recombined plasmid DNA sequences were found as newly replicated, high-molecular-weight DNA within the poxvirus-infected cells (Fig. VI-6 A). At 3 hrs post-transfection, transfected plasmid DNA was still found as unlinked, intact circles, although some conversion of form I to form II DNA was observed (Fig. VI-6 A, lanes 2 through 5). By 24 hrs, transfected plasmid DNA could be recovered only as a high molecular weight concatemer in excess of 40 kb in length (Fig. VI-6 A, lanes 6 through 9). Similar results were obtained in myxoma virus and vaccinia virus infected cells (data not shown). In the case of vaccinia virus infected cells, 1 order of magnitude less DNA was recovered than would be expected from the data shown in figure VI-4 B.

Essentially all of the recombined DNA was newly replicated, as shown by its resistance to *Dpn*I. Mammalian DNAs are not methylated at the *Dpn*I site, in contrast to DNAs purified from *dam*$^+$ E. coli cells (Peden *et al.*, 1980). The same DNAs used for the time course shown in figure VI-5 were digested with a combination of *Dpn*I and *Xho*I prior to electrophoresis and blotting. Prior to the onset of recombination in SFV infected cells, the bulk of recoverable plasmid DNA was input and was thus sensitive to cleavage with *Dpn*I (Fig. VI-6 B, lane 1). By 12 hrs post-transfection the plasmid DNA had been fully replicated and demonstrated resistance to cleavage by *Dpn*I (Fig. VI-6 B, lanes 2 to 4). Whether newly replicated DNA was selectively enriched in recombinants was also examined. At 10 hrs post-transfection, 46% of the detectable plasmid DNA was recombinant in the *Xho*I digested sample (Fig. VI-5, lane 4), whereas in the same DNA digested with *Xho*I and *Dpn*I, recombinant DNA forms composed approximately 61% of the *Dpn*I resistant *Xho*I fragments (Fig. VI-6 B, lane 3). This suggests that an excess of the recombinant products are present in newly replicated DNA.

Role of viral replication. The observations shown in figures VI-3, VI-4, and VI-6 suggest that recombination and viral DNA replication are closely associated events.

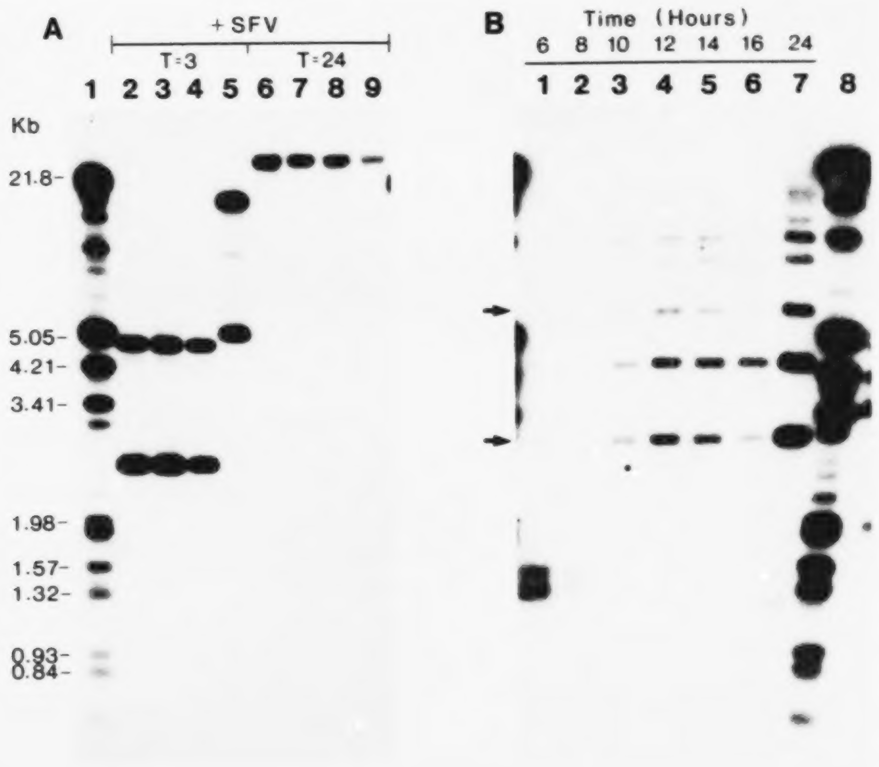


Figure VI-6. Recombinant products found within high-molecular weight, newly replicated concatemeric arrays in SFV-infected cells. SIRC cells were infected with SFV and transfected with the plasmid DNA indicated below. (A) DNA was recovered at the indicated times, fractionated and blotted without prior cleavage. pRDK35 (lanes; 2 and 6), pRDK39 (lanes; 3, and 7), pRDK35 plus pRDK39 (lanes; 4, and 8) and pRDK41 (lanes; 5 and 9). Size markers in lane 1 are λ DNA cleaved with *Eco*RI and *Hind*III. (B) Cells were transfected with pRDK41, and DNA was recovered at the indicated time points, cleaved with *Xho*I, fractionated, and Southern blotted as in Fig. VI-5. Note that by 10-12 hrs all the plasmid derived DNA, including the parental-sized fragments was newly replicated as indicated by resistance to cleavage with *Dpn*I. Arrows indicate the two *Xho*I fragment sizes (3.12 and 5.60 kb) of pRDK41.

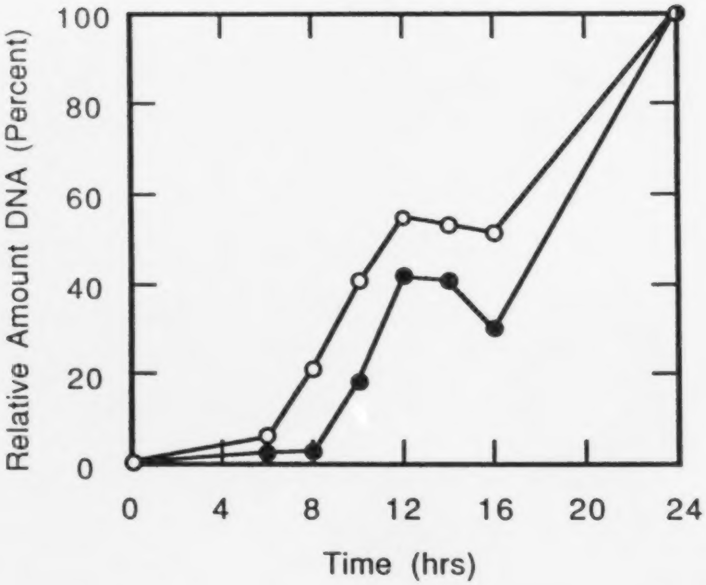


Figure VI-7. Approximately synchronous plasmid and viral DNA replication in SFV-infected cell. The autoradiograph shown in Fig. VI-6B was scanned with a densitometer, and the amount of newly replicated plasmid DNA was quantitated by integration of the densitometric trace. The amount of viral DNA was quantitated by densitometric analysis of a photograph of the original ethidium stained gel on which viral *Xho*I fragments were visible superimposed upon a uniform background of cellular DNA. In both cases, the amount of DNA present was normalized to the amount of plasmid or viral DNA found at 24 hrs. The scale is arbitrary, and does not reflect the fact that several orders of magnitude as much viral DNA is present by 24 hrs post-transfection as there is replicated-recombined plasmid DNA. Symbols: O, viral DNA; ●, plasmid DNA.

The initiation of viral and plasmid DNA replication were approximately coincident (Fig. VI-7) (see also DeLange & McFadden, 1986) and occurred 6 to 8 hrs post-transfection in SFV-infected cells in these experiments. This period was also precisely coincident with the onset of DNA recombination (Fig. VI-5).

To determine whether replication and recombination were inextricably linked, the effect of adding viral DNA polymerase inhibitors was examined by using SFV-infected cells transfected with pRDK35 plus pRDK39. The normal extent of recombination was determined by recovering DNA samples in the absence of drug at 6, 8, 10, 12, 14, 16, and 24 hrs. At each time point a parallel culture was washed, exposed to fresh medium containing 300 μg of PAA per ml (to inhibit the poxvirus DNA polymerase [DeLange & McFadden, 1986, Moss & Cooper, 1982]) and maintained until the 24 hr time point at which time the total DNA was recovered from each of the remaining cultures. Following digestion with *Xho*I, and Southern blotting (Fig. VI-8), scanning densitometry was used to quantitate the extent of recombination (Fig. VI-9). In these experiments, the time course showed that the onset of recombination occurred 6 to 8 hours post-transfection (Fig. VI-8, lanes 2, 5, Fig. VI-9 B). Addition of PAA at these times did not prevent the formation of recombinant molecules (Fig. VI-8, lanes 3, 6, Fig. VI-9 A), although it did greatly reduce the total amount of plasmid derived DNA that could be recovered relative to the amount recovered at 24 hrs from cells that had not been exposed to PAA. For example, only 12 % as much hybridizable plasmid DNA was recovered at 24 hrs from cells to which PAA had been added 6 hrs after transfection relative to that recovered from untreated cultures (Fig. VI-8 lanes 2, 3). We conclude from these experiments that viral DNA replication may not be an essential component of the recombination process *per se*, but at least some viral DNA polymerase activity must be present to maintain appreciable quantities of transfected DNA sequences. It should be noted; however, that since the kinetics of PAA inhibition have not been defined in this system, a requirement for low levels of DNA synthesis during the observed recombination can not be rigorously excluded. Interestingly the use of a different

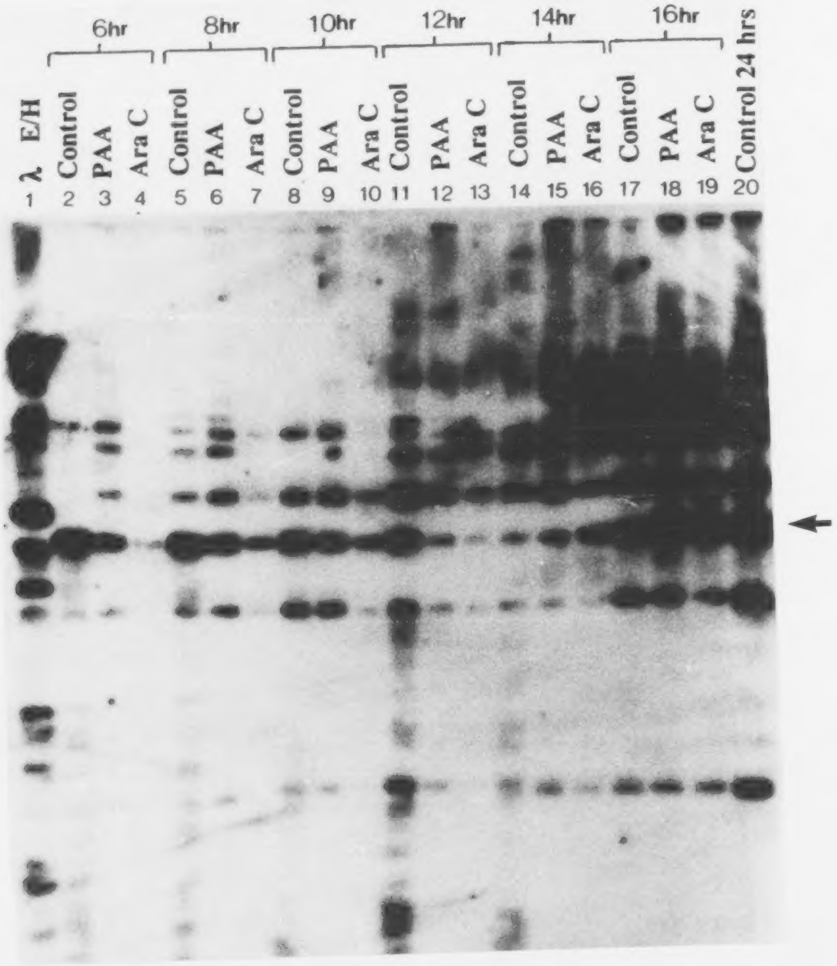

6hr
8hr
10hr
12hr
14hr
16hr
λ E/H
Control
PAA
Ara C
Control
PAA
Ara C
Control
PAA
Ara C
Control
PAA
Ara C
Control
PAA
Ara C
Control
PAA
Ara C
Control 24 hrs
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure VI-8. The effect of DNA synthesis inhibitors on poxvirus induced homologous recombination. Monolayers of SIRC cells were infected with SFV, and transfected with a combination of the plasmids pRDK35 and pRDK39. At the times indicated the samples in the control lanes were harvested (lanes; 2, 5, 8, 11, 14, 17, and 24) at each of the time points indicated identically infected transfected monolayers were washed and had their medium replaced with medium containing either 300 μg/ml PAA (lanes; 3, 6, 9, 12, 15, and 18), or 40 μg/ml Ara C (lanes; 4, 7, 10, 13, 16, and 19). Each of these samples was then incubated until the 24 hr time point and then harvested. All samples were then cleaved with *Xho*I, fractionated, and Southern blotted. A λ *Eco*RI-*Hind*III digest was used as a size standard (lane 1). The amount of recombination that occurred following the addition of drug can be determined by comparing the amount of recombinant fragments in the control and the drug treated lanes at each time point. The unrecombined 4.36 Kb parental DNA is indicated by the arrow. Other bands are indicative of the formation of recombinant plasmid species

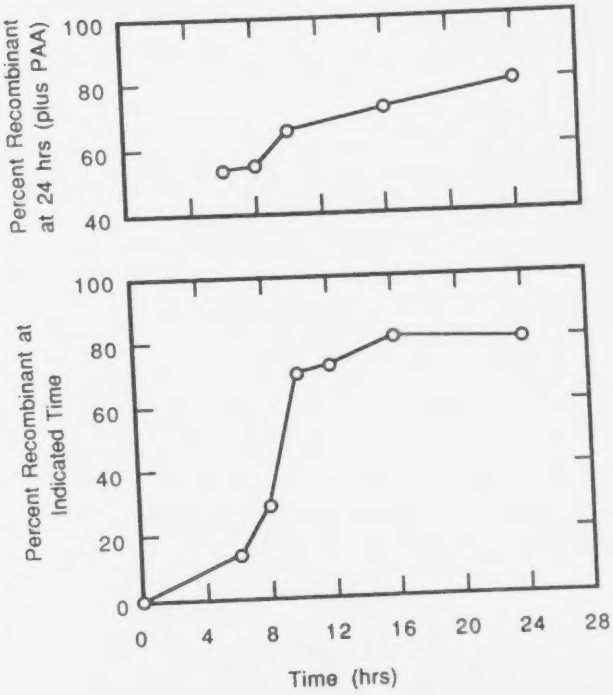


Figure VI-9. A graphic representation of the effect of PAA on recombination of transfected plasmids in SFV-infected cells. The autoradiograph in figure VI-8 was densitometrically scanned to determine the extent of recombination that occurred in drug treated and untreated cells. (A) The fraction of plasmid DNA that can be shown to be recombinant at the time indicated. Note that in cells that were continuously exposed to PAA, recombination still continued. The percent plasmid DNA found in recombinant forms goes from 16% at 6 hrs (panel B) to 55% 18 hrs later (panel A) in the presence of PAA, although further amplification of both viral and plasmid sequences was largely blocked.

DNA synthesis inhibitor (ara C) had a much more stringent effect on total DNA synthesis and upon the production of recombinant molecules (Fig. VI-8, lanes 4, 7, and 10).Very few recombinants were generated when ara C was applied earlier than 10 hrs post-transfection (Fig. VI-8, lane 10). No effect upon the formation of recombinants was observed when the DNA synthesis inhibitors were added at times later than 10 hours post-transfection, by this time most of the recombinant structures had already been formed. The most likely explanation for these results is that PAA is a much less effective inhibitor of the SFV DNA polymerase than ara C is.

Inhibitors of protein and RNA synthesis have much less stringent effects upon the formation of recombinant molecules in SFV infected cells than do inhibitors of DNA synthesis (Fig. VI-10). Total DNA that was collected 24 hrs post-infection after the addition of 100 µg of cycloheximide per ml medium displayed a considerable amount of recombinant molecule formation (Fig. VI-10 lane 2) even though the total amount of DNA was less than in the absence of drug (compare with Fig. VI-10, lane 1). A somewhat more drastic effect was observed when an inhibitor of RNA synthesis was applied to the infected/transfected cells at 6 hrs post-transfection (Fig. VI-10 lane 3). It is noteworthy that in this case as well as at the later time point (Fig. VI-10, lanes 5 and 6) apparently equal amounts of the parental form of plasmid DNA was present in the cycloheximide and actinomycin D treated lanes but the actinomycin D treatment resulted in a slightly decreased formation of recombinant molecules. Neither of these drugs had a demonstrable effect on the formation of recombinant molecules if they were added to the infected cell cultures at times latter than 10 hrs post-transfection. These results strongly suggest that all of the poxviral gene products that are required to catalyze homologous recombination within the infected cell cytoplasm are synthesized within the first 4 to 6 hours of the infection. None of the plasmids that were used in this study possess a known viral promoter so it is not clear why an inhibitor of RNA synthesis should have a more drastic effect upon the recombination of these plasmid molecules than an inhibitor of protein synthesis. One

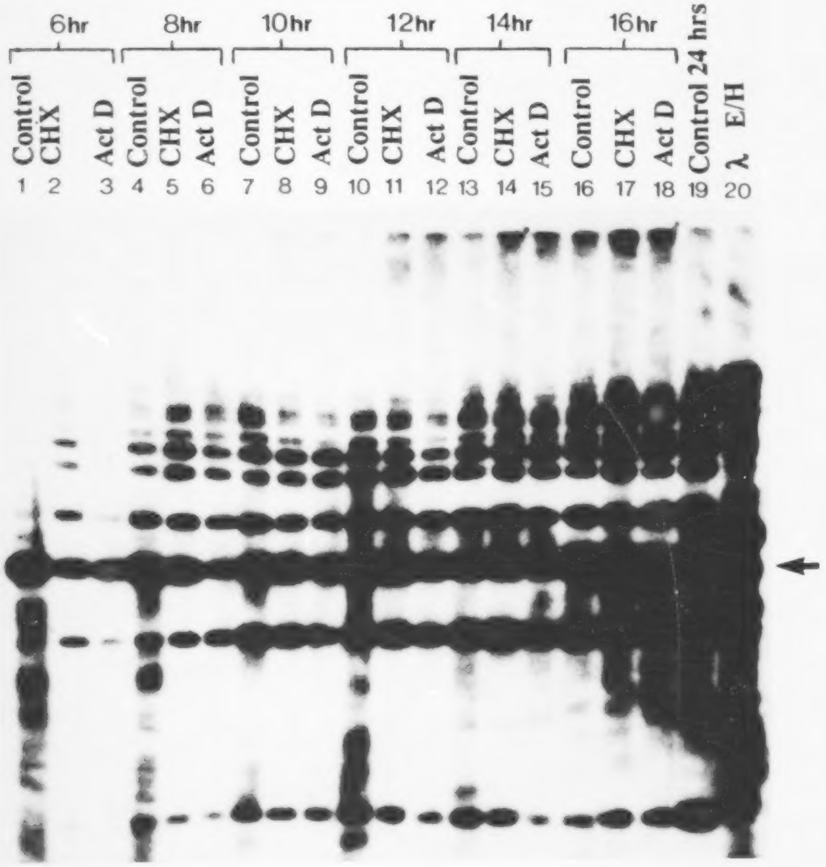

6hr
8hr
10hr
12hr
14hr
16hr
Control 1
CHX 2
Act D 3
Control 4
CHX 5
Act D 6
Control 7
CHX 8
Act D 9
Control 10
CHX 11
Act D 12
Control 13
CHX 14
Act D 15
Control 16
CHX 17
Act D 18
Control 24 hrs 19
λ E/H 20

Figure VI-10. The effect of inhibitors of RNA and protein synthesis on the recombination of transfected plasmid DNA in SFV infected cells. Similar to the experiment illustrated in Fig. VI-8, monolayers of SIRC cells were infected with SFV and then transfected with a mixture of plasmid DNA pRDK35 and pRDK39. The total DNA was harvested from the control samples at the time points indicated and at each of these time points identically infected/transfected samples were treated with either 100 μg/ml cycloheximide (CHX) or 10 μg/ml actinomycin D (Act D). All of the drug treated samples were harvested at 24 hrs and the DNA was cleaved with *Xho*I, fractionated and Southern blotted. Size standard is a λ *Eco*RI-*Hind*III digest (lane 20). The position of the unrecombined 4.36 Kb parental DNA is indicated by the arrow.

plausible explanation is simply that the presence of actinomycin D is inhibitory by direct interaction with the recombination enzymes, another possibility is that actinomycin D is inhibitory by virtue of its ability to intercalate into and positively supercoil the plasmid DNA potentially making it a less efficient substrate for the recombination reaction.

The observed products are true recombinants. The observation that transfected DNAs can be subjected to extraordinarily high levels of mutation (Calos *et al.*, 1983, Razzaque *et al.*, 1983, Thomas & Capecchi, 1986) makes caution mandatory when assuming that the products detected by the blotting procedure are true recombinants. For example, mutation of the *Xho*I sites could potentially generate some, although not all, of the observed products. This seems unlikely, given the controls shown in figures VI-3 and VI-4, in which infected cells transfected with pRDK35 or pRDK39 alone retained only the input plasmid DNA restriction pattern. However, to confirm the validity of the quantitation method, the recombination frequency was calculated by transforming the recombinant DNA recovered from infected/transfected SIRC cells back into rec A E. coli and measuring the proportion of transformants that were tetracycline resistant relative to the total number of ampicillin-resistant transformants (Table VI-1).

Recombination frequencies measured by the transformation method underestimate the true recombination frequency, since only a subset of the recombinant products are tetracycline resistant. In general only 25% of the recombinants measured by densitometry are Tet^r. This is best illustrated by considering the hypothetical case of quantitatively recombined DNA in which the distribution of *Xho*I sites has been completely randomized. Only 25% of the pBR322 episomes would be Tet^r, although 100% are recombinant. Taking this correction into account, it is clear from table VI-1 that transformation assays were also able to detect the products of high frequency *in vivo* recombination and that quantitatively, transformation and Southern blot assays gave almost identical results. This transformation assay was not sensitive enough to detect the lower level of recombinants

		E. coli transformation method[a]				Blotting method[b]	
Infection	time (hr) post-transfection	No. AMP^{r}[c]	No. patched	No. AMP^{r} TET^{r}	%TET^{r}	% Recombinant	% TET^{r}
None	3	12,104	150	0	<1	ND[d]	0
	24	86	41	0	≤2	ND	0
SFV	3	5,140	150	0	<1	ND	0
	24	686	148	26	18	57	14
Myxoma	3	14,300	150	0	<1	ND	0
	24	1,120	150	21	14	61	15
Vaccinia	3	9,688	150	0	<1	ND	0
	24	171	39	0	≤3	25	6

Table VI-1. Comparison of recombination frequencies measured by Southern blotting and E. coli transformation assay (this experiment was performed and the data compiled by Dr. D. H. Evans). [a]DNA was recovered from cells transfected with a mixture of pRDK35 and pRDK39. The recovered DNA was digested with *Eco*RI, circularized, and transformed into E. coli. The percent recombination was calculated as described in the Materials and methods. [b]The blotting method was quantitated by densitometric analysis of autoradiographs. [c]Number of transformants per microgram of DNA. [d]ND, not detectable.

formed in vaccinia virus infected cells, although statistically the result is not in conflict with frequencies expected on the basis of the Southern blot data. Both SFV and myxoma virus infections; however, induced statistically significant ($P \geq 99.5\%$) increases in the recovery of amp^r, tet^r recombinants, in excellent agreement with the data from Southern blot analyses. Thus, we can conclude that the novel plasmid *Xho*I fragments detected by blotting are an accurate reflection of extremely high levels of *bona fide* genetic recombination in these poxvirus-infected cells.

D. Discussion

The use of DNA substrates containing polymorphic restriction sites has proven very useful in experiments designed to investigate the process of homologous recombination in a number of organisms both *in vivo* and *in vitro* (Doherty *et al.*, 1983, Shapiro *et al.*, 1983). Poxviruses provide a unique model system for the study of general recombination because, their cytoplasmic site of DNA replication is distinct from the site where the events of host nuclear replication and recombination occur. By virtue of this autonomous mode of replication poxviruses are believed to encode most of the enzymatic machinery for viral RNA and DNA synthesis (reviewed by Moss, 1990, Traktman, 1990). It has been shown elsewhere that when exogenous circular plasmid DNAs are transfected into poxvirus infected cells, the input DNA is replicated by a mechanism under the control of viral *trans*-acting factors (DeLange & McFadden, 1986). The replication process takes place in the cytoplasm of poxvirus infected cells resulting in the production of high molecular weight concatemeric arrays of plasmid DNA (DeLange & McFadden, 1986) that are not packaged into nascent virions. One exception to this situation is plasmid DNA that contains a cloned version of a poxviral telomere replicative intermediate and the sequences necessary for the resolution of the replicative intermediate. In this case the replicated DNA is recovered as a ladder of monomer and multimer linear minichromosomes with viral hairpin termini

(DeLange *et al.*, 1986, Merchlinsky & Moss, 1986). In this study we show that plasmids containing polymorphic *Xho*I restriction sites can be used to demonstrate that this amplification of transfected plasmid sequences is associated with the induction of extremely high levels of genetic recombination activity in the cytoplasm of the infected cells.

The general recombination that we observe among the transfected plasmid DNA sequences is strictly dependent upon the presence of the infecting poxvirus. By using either a blotting method to detect recombinant plasmid *Xho*I restriction fragment sizes or a transformation assay to detect the regeneration of a wild type tetracycline resistance gene, recombination was not detectable within the uninfected SIRC cells within the 24 hr span of the experiments presented here. It is possible that the rabbit SIRC cells used in this study were less proficient at recombination than many of the rodent or primate cell lines that have been used in other studies. However, it is more likely that the transient assay used here is less sensitive to nuclear recombination events than are other recombination assays because the parameters have been optimized specifically to assess cytoplasmic recombination events. It may simply be the case that the nanogram quantities of substrate DNA and lack of carrier DNA minimize the amount of plasmid that enters the uninfected cell nucleus and is subjected to the host recombination activities. Likewise, the small amount of plasmid DNA that is recombined within the uninfected cell may not be detectable by our protocol.

In this study the amount of homologous recombination induced is virus specific and follows the order: myxoma virus ≅ SFV > vaccinia virus. This order also matches the observed capacity of these three viruses to catalyze sequence non-specific replication of transfected DNAs (DeLange & McFadden, 1986). This result is somewhat surprising given that a similar set of plasmids was used to study intramolecular recombination in vaccinia virus infected cells and in this case the plasmid DNA was replicated and recombined very efficiently (Merchlinsky, 1989). The transfection protocols may explain some of the differences between these two studies. We transfected only 50 ng of plasmid DNA 2 hrs post-infection while in the latter study 800 ng of plasmid DNA was transfected 30 mins post-infection. Other small differences such as the multiplicity of infection; and the use of different cell lines could potentially affect the observed result. All three poxviruses replicate well in SIRC cells, as can be shown by calculating the virus yield from a one step growth curve and by examining the relative levels of virus specific restriction fragments on ethidium bromide stained agarose gels (Fig. VI-3 A and VI-4 A). While this eliminates poor levels of vaccinia virus replication in SIRC cells as a trivial explanation of this observation it remains possible that by transfecting the plasmid DNA 2 hrs after the infection that the plasmid DNA is in some way excluded from the recombination activity in the rapidly growing vaccinia virus "factory". The fact that infection with different viruses has different effects on the amount of induced cytoplasmic replication and recombination further implicates viral gene products in the induced activities. Although this is an attractive idea, other experiments must be performed before such a conclusion can be verified. It could always be argued, for example, that the presence of replicating viral DNA or some specific viral gene products induces the expression of host cell functions that subsequently catalyze plasmid recombination and that the two Leporipoxviruses are more proficient at this induction than is vaccinia virus. Thus concrete evidence that it is indeed viral gene products that catalyze the cytoplasmic recombination events will require the isolation of the viral gene products responsible or the isolation of viral mutants that are specifically defective in homologous recombination processes.

The observed frequency of recombination in poxvirus-infected cells appears to be very high. Of the pBR322-derived plasmid DNAs recovered from SFV- or myxoma virus-infected cells, 18% were Tet^r, which approaches the theoretical limit (assuming no bias in the directionality of gene conversion) of 25%. This indicates that the two *Xho*I markers have, by 24 hrs been segregated on a virtually random basis, to the extent that further rounds of recombination can not be measured. Quantitatively this agrees quite well with the frequency calculated from Southern blots, assuming that all *Xho*I resistant plasmid species of ≥ 7.48 kb contain a functional tetracycline resistance gene. This suggests that high frequencies of mutagenesis are not involved in generating the results quantitated by blotting (Table VI-1). A minimal recombination frequency can be estimated from a Poisson analysis of the *Xho*I fragment distribution and suggests that at least one recombination event takes place per 7.1, 8.0, and 45 kb of transfected DNA in SFV-, myxoma virus-, and vaccinia virus- infected cells, respectively. How this compares with viral recombination frequencies is more difficult to assess, since detailed comparisons of the genetic and physical maps of all three viruses have not been made. Ensinger & Rovinsky (1983) and Drillen & Spehner (1983) have mapped temperature-sensitive mutations in vaccinia virus with respect to both the genetic and the physical maps. They obtained a minimal recombination frequency of between 0.9% and 1.8% per 1 kb (or one event per 55 to 110 kb), which is somewhat lower than the plasmid frequency that we have observed between transfected plasmids in vaccinia virus-infected cells. This suggests that whatever the mechanism of recombination, transfected plasmid and endogenous plasmid DNA may be subjected to the same processes, although the endogenous replicating viral genomes may be less accessible to the induced recombination activities than naked transfected DNAs are.

One of the most significant findings of this study is the close association of viral DNA replication and recombination. The original observation that transfected plasmid DNAs can be recovered from poxvirus-infected cells as high-molecular-weight concatemers specifically in the head-to-tail orientation suggested that either a rolling circle mode of DNA replication or a high level of generalized recombination was taking place (DeLange & McFadden, 1986). The two events may be associated processes, given the significant excess of recombinants in newly replicated DNA and the roughly coincident timing of replication and recombination (Fig. VI-7), but whether the two processes are inextricably linked is difficult to deduce from this data. In a similar study using a battery of vaccinia virus temperature-sensitive mutants it was determined that no detectable plasmid intramolecular recombination occurred in the absence of plasmid DNA replication (Merchlinsky, 1989). A similar conclusion was drawn when viral DNA synthesis was inhibited by PAA (Merchlinsky 1989). Inhibition of the SFV DNA polymerase with PAA did not entirely inhibit subsequent intermolecular and intramolecular recombination events, although it is noteworthy that in the presence of PAA a significant amount of plasmid DNA amplification occurred. Thus the Leporipoxvirus DNA polymerase may have a greater resistance to PAA than does the Orthopoxvirus DNA polymerase. The results with ara C demonstrate a more stringent inhibition of viral DNA replication with a subsequent essentially complete elimination of plasmid recombination. Similar conclusions have been drawn from experiments investigating intermolecular and intramolecular homologous recombination in vaccinia virus DNA. Ara C or hydroxyurea added early in the infection blocked the formation of recombinants in viral DNA. This same study demonstrated that addition of actinomycin D or cycloheximide to infected cell cultures prior to the initiation of viral DNA replication blocked recombination (Ball, 1987). All of these results are consistent with a requirement for DNA synthesis in poxvirus induced homologous recombination. Furthermore the addition of cycloheximide or actinomycin D after viral DNA replication was in progress (8-10 hrs post-infection) failed to block both the amplification and recombination of exogenously added plasmid sequences. These results are also consistent with the finding that only early gene products are required to catalyze homologous recombination (Merchlinsky, 1989); by blocking protein synthesis at 6 hrs post-infection we still saw recombinant formation and plasmid amplification, while it has been observed that the addition of cycloheximide or actinomycin D 2 hrs post-infection blocks the formation of recombinants (Ball, 1987). The linkage of virally induced recombination with DNA replication is not entirely without precedent. It has been demonstrated that homologous recombination catalyzed in HSV infected cells is dependent upon replication of the substrate DNA by the viral DNA replication machinery (Weber *et al.*, 1988).

No poxvirus mutants have been identified that display a specific defect in homologous recombination (Merchlinsky, 1989); however, the observation that all mutants displaying a defect in DNA synthesis are also defective in general recombination suggests that DNA synthesis is tightly linked with recombination, and that some parts of the replication apparatus may function in recombination. No viral gene products have been identified that have a clear role in DNA recombination with the exception of DNA polymerase. It has been suggested that the viral DNA polymerase serves a function in recombination other than DNA synthesis (Colinas *et al.*, 1990). While enzymes that serve multiple functions seem to be relatively common in poxviruses, it is difficult to assess the validity of the claim. The work by Colinas *et al.*, (1990) demonstrated that PAA and ara C inhibit replication and recombination of plasmid DNA; however in the same study hydroxyurea (HU) was shown to actually stimulate plasmid replication and recombination above the level observed in an infection performed in the absence of drugs. At the same time it was shown that HU absolutely inhibited the synthesis of viral DNA sequences. A similar result was presented for the vaccinia virus mutant ts25; that is transfected plasmid DNA was replicated and recombined at the nonpermissive temperature but the viral DNA is not replicated. The study of Colinas *et al.*, (1990) examined intermolecular recombination. One aspect of the plasmid substrates that was unique to the study was that they contained poxvirus early/late promoters and are thus expected to be transcribed throughout the course of the infection. It is difficult to assess the significance of transcription in this context but it has been demonstrated that active transcription of DNA sequences stimulates homologous recombination of those sequences (Thomas & Rothstein, 1989, Nickoloff & Reynolds, 1990).

We, and others have thus demonstrated a tight linkage between poxviral DNA replication and virus induced general recombination. It remains to be determined whether the two events can be rigorously segregated. Recombination may very well turn out to be an important component of poxvirus replication, as has been observed in bacteriophages such as T4, or may simply be a useful byproduct of the mode of replication that allows for genetic diversity, and repair of lesions to the genomic DNA.

Finally, given the observation that plasmid recombination frequencies are at least comparable to viral recombination frequencies and that the plasmid DNAs seem to function as templates for the *trans*-acting viral activities required for replication, recombination and telomere resolution. It is reasonable to speculate that transfected DNAs are subjected to the same recombination processes as are the viral DNAs. Transfected plasmid constructs should in turn prove to be useful probes for studying the process of poxvirus recombination, and poxvirus-infected cells may turn out to be an ideal source of the gene products that catalyze these events.

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Chapter VII

Identification, Expression and Characterization of Uracil DNA Glycosylase Encoded by Shope fibroma virus: A DNA repair enzyme that is essential for viral replication

A. Introduction

Most organisms safeguard their genetic material with several specific mechanisms for the repair of damaged or incorrectly incorporated bases (Friedberg 1985). DNA glycosylases act in the initial stage of the pathway for excision repair of damaged bases by catalyzing the hydrolysis of the damaged base at the N-glycosylic bond (Duncan, 1981). Uracil DNA glycosylases remove uracil residues that have been introduced into DNA either through misincorporation of dUTP by DNA polymerase or through the deamination of cytosine (Lindahl & Nyberg, 1974). The former event is not necessarily mutagenic leading to an A:U basepair and occurs with low frequency owing to the low cellular pools of dUTP (Shlomai & Kornberg, 1978, Goulian *et al.*, 1980). However, the latter event causes a G:U pairing that can result in a G:C to A:T base pair substitution if not repaired prior to DNA replication. A family of DNA glycosylases with different substrate specificities allows cells to convert many different kinds of damage into a single lesion-an apurinic-apyrimidinic (AP) site. The AP site can be broken by AP-endonucleases and then repaired by a combination of DNA polymerase and DNA ligase (Friedberg, 1985). The significance of a pathway for uracil removal is evinced by the apparently ubiquitous nature of uracil DNA glycosylases. To date all prokaryotic and eukaryotic organisms that have been screened have been found to express at least one form of uracil DNA glycosylase (reviewed by Friedberg, 1985, Sakumi & Sekiguchi, 1990). Most eukaryotic organisms have a nuclear and an organellar form of the enzyme (Anderson & Friedberg, 1980, Gupta & Sirover, 1981, Domena & Mosbaugh, 1985). Until recently mammalian herpes viruses were the only eukaryotic viruses that had been reported to encode a uracil DNA glycosylase (Worrand & Carradonna, 1988). Infection with HSV 1 or 2 results in a rapid induction of both dUTPase and uracil DNA glycosylase (Carradonna & Cheng, 1980). The induction of these activities may be necessary in order to maintain the integrity of the viral DNA during a period of rapid DNA synthesis in the presence of greatly expanded cellular pools of DNA precursors (Jamieson & Bjursell, 1976).

We have recently identified a gene encoded by the Shope fibroma virus (SFV) that bears considerable homology to known uracil DNA glycosylases (Upton *et al.*, 1991 unpublished observation). SFV is a member of the Leporipox family of poxviruses and is related to the more well studied Orthopoxvirus vaccinia virus. Poxviruses are large DNA viruses that replicate in the host cell cytoplasm independent of any host nuclear functions (Hruby *et al.*, 1979). By virtue of their cytoplasmic site of DNA synthesis, poxviruses are able to overcome cellular regulatory functions but must encode all of their own replicative and transcriptional enzymes. Previous studies have reported a lack of mismatch repair activities in SFV infected cell cytoplasms (Fisher *et al.*, 1991), and in fact unpaired bases are a conserved feature of the termini of poxvirus genomes (DeLange & McFadden, 1990). It was of interest therefore to discover that poxviruses encode an enzyme that functions in the initial phase of a DNA excision repair pathway.

In this paper we report some aspects of the expression of the poxviral uracil DNA glycosylase and present the expression and characterization of a recombinant form of the enzyme synthesized in E. coli.

B. Materials and Methods

Fluoresence assay for Uracil DNA glycosylase. The fluoresence loss assay that was used to detect uracil DNA glycosylase activity has been described previously (Evans *et al.*, 1984, Morgan & Chlebek, 1989). This assay is dependent upon the enhanced fluoresence of ethidium bromide following intercalation into duplex DNA. At pH 12 long duplex DNAs are stabilized by the intercalation of ethidium bromide while shorter duplexes are denatured with a subsequent loss of fluoresence. Following thermal denaturation at 95°C covalently closed DNA will rapidly reanneal and regain all of its characteristic fluoresence whereas the strands of a nicked DNA molecule will separate and be prevented from reannealing by the high pH condition (Fig. VII-1a). Thus the loss of fluoresence that occurs following heating can be used to monitor the creation of nicks in DNA. The treatment of uracil containing DNA with a uracil DNA glycosylase results in the production of AP sites where uracil residues have been excised. The phosphodiester backbone of the DNA containing AP sites remains intact, thus, no alteration in fluoresence is observed (Fig. VII-1c). However, AP sites are sensitive to hydrolysis under alkaline conditions so heating the (AP) DNA to 95°C for 5 minutes results in strand breakage at AP sites with subsequent irreversible denaturation that is manifest as a loss of fluoresence at pH 12 (Fig. VII-1c). Standard assay reactions for uracil DNA glycosylase activity contained 50 mM Tris-HCl pH 7.5, 20 mM EDTA, 100 µg/ml heat denatured gelatin and 1 µg of substrate DNA in a 50 µl volume. A modification of these conditions was used to assay the formation of AP sites in the substrate DNA (Evans *et al.*, 1984). The uracil DNA glycosylase reactions were done under the same conditions; however, instead of adding the samples directly to the pH 12 assay buffer and heating to 95°C, the samples were divided into two aliquots. One half of the sample was added directly to the pH 12 assay buffer and the fluoresence was determined. The other half of the sample was added to 2 volumes of deionized Me_2SO. This treatment causes denaturation of nicked DNA but has no effect upon the fluoresence of DNA that contains AP sites (Fig VII-1d) (Evans *et al.*, 1984). By heating this DNA to 95°C, measuring the fluoresence and then comparing with the fluoresence change caused by Me_2SO treatment alone it was possible to determine if AP sites had been generated (Fig. VII-1d). In this way it was possible to distinguish between the formation of AP sites and nicking of the DNA.

Preparation of substrate DNA. The uracil containing substrate DNA that was used for these studies was prepared by transforming the dut^-, ung^- E. coli strain CJ236 (Kunkel *et al.*, 1987) with pUC 19. The plasmid DNA generated from alkaline lysis of 200 ml cultures

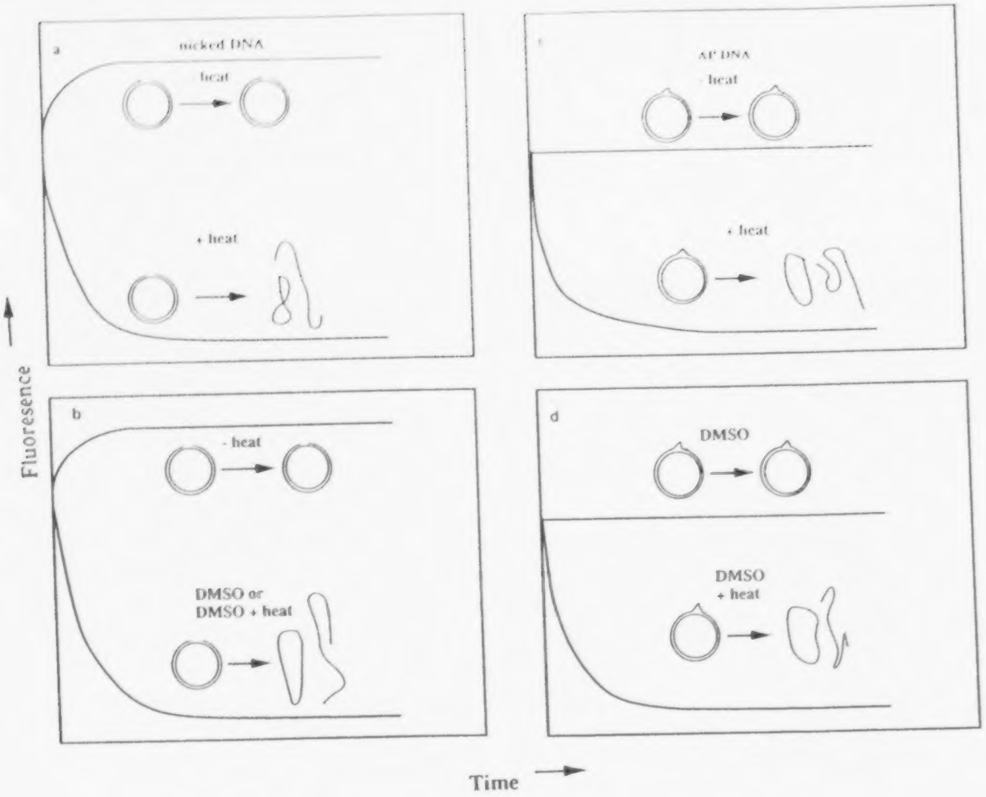


Figure VII-1. Idealized curves summarizing fluoresence assays that detect the formation of nicks or AP sites in a circular DNA substrate. (a) The fluoresence of DNA as it becomes nicked by an endonuclease slowly rises over time in the absence of heat; however when the sample is heated to 95^{o}C the DNA strands separate with a subsequent loss of fluoresence. (b). The introduction of nicks can be similarly detected by denaturing the sample with Me$_2$SO. (c) AP sites that are introduced into DNA by the action of a DNA glycosylase do not affect the fluoresence of unheated DNA. Heating the AP DNA to 95^{o}C hydrolyzes the AP sites allowing the DNA strands to separate with a subsequent loss of fluoresence. (d) In the presence of Me$_2$SO, AP sites remain intact and thus the fluoresence of the DNA remains unchanged while the fluoresence of nicked DNA decreases as the strands separate due to denaturation. Heating the DNA causes hydrolysis of the AP sites leading to a subsequent loss of fluoresence.

of the transformant was purified by isopycnic banding on CsCl gradients. Using this method it was possible to generate very large quantities of uracil-containing substrate DNA without the use of any mutagenic chemicals. One draw back to this procedure for generating uracil containing DNA is that it is not possible to control the amount of uracil incorporated into each target molecule.

Cells and viruses. The cells used in this study; BGMK (African green monkey) and SIRC (rabbit corneal), were obtained from the American Type Culture Collection (ATCC), and were propagated as monolayer cultures in Dulbeco's modified Eagle's medium (DME) supplemented with 10% newborn calf serum (Gibco laboratories). SFV was obtained from the ATCC and was propagated by infection of monolayers of $3x10^8$ BGMK cells with virus at a multiplicity of 1 focus forming unit (ffu) per cell. The infections were allowed to proceed for 48 hrs at which time the monolayer was harvested by incubating the cells in 1x SSC (150 mM NaCl, 15 mM sodium citrate pH 7.2) for 15 mins followed by rapid shaking. The detached cells were concentrated by centrifugation washed in PBS (145 mM NaCl, 4 mM KCl, 10 mM phosphate pH 7.3), and then allowed to swell in 10 mM Tris-HCl pH 8.0 on ice. Virus was released from the swollen cells by multiple stokes of a Dounce homogenizer and the cell debris was removed by low speed centrifugation. The virus in the supernatant was concentrated by centrifugation for 60 min at 25,000xg. Virus titres were determined by infecting monolayers of $1x10^6$ BGMK cells with serial 10 fold dilutions of the virus stocks. The infections were allowed to proceed for 5 days, and then the cells were fixed, stained with 0.1% crystal violet and the foci were counted.

The induction of uracil DNA glycosylase activity was determined by assaying crude cytoplasmic extracts made from monolayers of $1x10^6$ SIRC cells that had either been mock infected or infected with SFV at a multiplicity of 10 ffu/ cell. The monolayers were harvested by first washing the cells in cold PBS+ 5 mM EDTA and then scraping up the monolayers in 300 µl of cold PBS. The harvested cells were pelleted by centrifugation at 12,000 rpm in a microfuge and the cell pellet was resuspended in 100 µl of 10 mM Tris-

HCl pH 8.0, 10 mM EDTA, 0.2% NP-40. The cells were incubated on ice for 15 mins and then lysed mechanical breakage, the nuclei and cell debris were pelleted by low speed centrifugation and the cytoplasms were snap frozen in a dry ice-ethanol bath and stored at -70°C.

Two E. coli strains were used for the expression of the SFV uracil DNA glycosylase gene. CJ236 (Kunkel *et al.*, 1987) which has mutations in the dUTPase gene (dut$^-$) and the uracil DNA glycosylase gene (ung$^-$), and BL21 (DE3) (Studier & Moffat, 1986) which is protease deficient and carries the phage T7 RNA polymerase under the control of an inducible Lac UV promoter.

PCR amplification and Northern blot analysis. The coding sequence for the SFV D6 open reading frame was obtained by PCR amplification from SFV genomic DNA that was prepared as described (Esposito *et al.*, 1981). Two oligonucleotide primers were synthesized that were complementary to the 5' and 3' ends of the D6 coding sequence. To facilitate cloning of the amplified sequence the 5' oligonucleotide incorporated a unique *Nde*I restriction enzyme site into the primer; 5'-GGCATATGAGACGGGTATT-3', and the 3' oligonucleotide incorporated a unique *Bam*HI site; 5'-GGGGATCCTATTGATTTTAAAACA-3'. The D6 open reading frame was amplified by 20 cycles of 96°C for 30 secs, 55°C for 45 secs, and 72°C for 45 secs. The amplified 660 bp fragment was purified by preparative gel electrophoresis (Langridge *et al.*, 1980) and separated into two aliqouts. One half of the sample was labelled with α [^{32}P] dATP by random priming and extension of the primers with the Klenow fragment of DNA polymerase I (Feinberg & Fogelstein, 1984). The labelled DNA was then purified by extraction with an equal volume of phenol:chloroform (1:1) and chloroform followed by precipitation with ethanol, 2 washes with 70% ethanol and finally resuspended in 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

RNA for Northern blots was prepared by infection of monolayers of 3.5x10^6 BGMK cells in 100 mM dishes with SFV at a multiplicity of 10 ffu/cell. The infections were allowed to proceed for 16 hours to isolate late RNA or were allowed to proceed for 16 hrs in the presence of 100 μg/ml cycloheximide to collect RNA from genes expressed exclusively prior to DNA replication (early RNA). The total nucleic acids were collected in both cases by lysing the cells on the plates by the addition of a 2.5 ml solution of 6 M guanidinium isothiocyanate, 0.5% sarkosyl, 0.1M β-mercaptoethanol, and 5 mM sodium citrate pH 7.0. The cell lysate was scraped from the plate and DNA was sheared by 5 passages through a 26 gauge needle. To each 2.5 ml of homogenate 1 g of solid $CsCl_2$ was added and this lysate was layered on to a 2.5 ml cushion of 5.6 M $CsCl_2$, 100 mM EDTA and the RNA was pelleted by centrifugation at 37,000 rpm in an SW50.1 rotor for 17 hours at 20°C. The pelleted RNA was resuspended in diethylpyrocarbonate treated water, extracted 3 times with chloroform, and then precipitated with ethanol. 10 μg of the early or late total RNA was loaded into each lane of a 1% agarose, 2.2 M formaldehyde gel and fractionated by electrophoresis (Sambrook *et al.*, 1989). Following capillary transfer to a hybond-C membrane (Amersham) and U.V. crosslinking the RNA was hybridized with the labelled D6 probe and the blots were visualized by autoradiography.

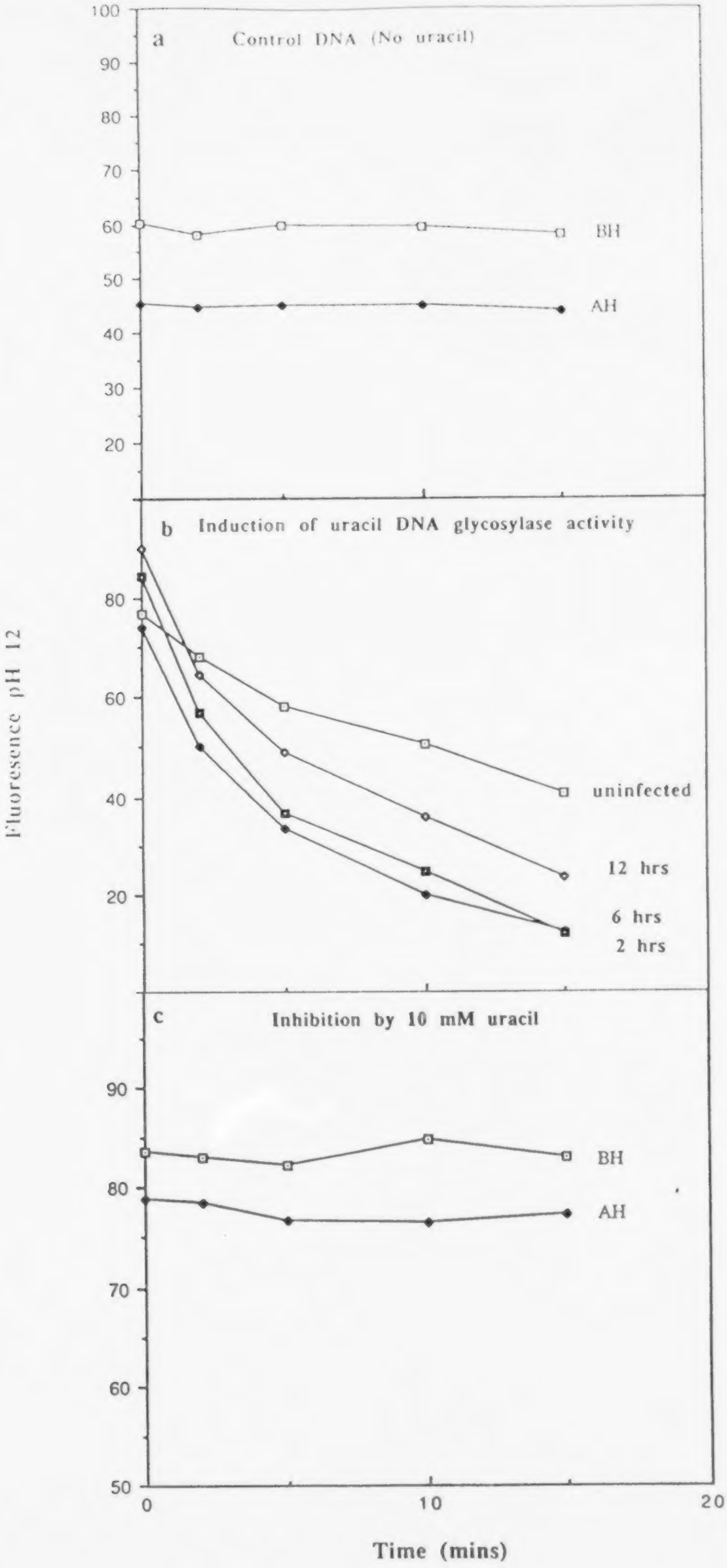
A second aliquot of the PCR product was cleaved with restriction enzymes *Nde*I and *Bam*HI, and ligated into the *NdeI*/ *Bam*HI site of the expression vector pET-3a (Rosenberg *et al.*, 1987). This ligation was used to transform E. coli HB101 and the DNA from ampicillin resistant colonies was screened for the correct insert by cleavage with *Nde*I and *Bam*HI followed by fractionation of the digestion products on an 8% nondenaturing polyacrylamide gel. Three of the clones containing inserts of the expected size were subjected to DNA sequencing to confirm the nature of the insert. In each case the insert sequence was identical to that obtained in the initial sequencing of the D6 open reading frame (Upton *et al.*, 1991 [submitted]). One of the correct clones pXSUG-1 was used as a target DNA for an *in vitro* transcription reaction. 1 μg of the plasmid DNA was linearized with *Bam*HI which cleaves uniquely at the 3' end of the coding sequence. This DNA was then purified by extraction with equal volumes of phenol, phenol:chloroform (1:1), and chloroform, then precipitated with ethanol and resuspended in 5 µl of sterile 10 mM Tris-HCl pH 7.5, 1 mM EDTA. *In vitro* transcription reactions were conducted in 40 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1 mM spermidine in the presence of 500 µM each of ATP, CTP, UTP,and 50 µM GTP, 500 µM mG(5')ppp(5')G cap analog. 4 U of T7 RNA polymerase (Pharmacia) were added and the reactions were incubated at 37°C for 1 hr. The target DNA was then degraded by the addition of 1 U RNase-free DNase (Promega) and the RNA was purified by extraction with phenol:chloroform (1:1) and chloroform, followed by precipitation and resuspension in RNase free water. This RNA was used to program a nuclease treated rabbit reticulocyte lysate (Promega) in the presence of [^{35}S] methionine (ICN radiochemicals). The translation products from a programmed and an unprogrammed lysate were assayed directly in the pH 12 fluoresence assay and an aliquot of each was analyzed by electrophoresis through a 12% SDS polyacrylamide gel. Following electrophoresis, molecular weight markers were visualized by staining the gels with Coomassie blue and then destaining with 10% methanol, 10% acetic acid. The gel was then dryed and autoradiographed to visualize the translation products.

The clone described above was also used to transform E. coli strains CJ236 and BL21 (DE3). Expression of the D6 open reading frame from the phage T7 promoter sequence in pXSUG-1 was induced in CJ236 by transforming the cells with a second plasmid pGP2-1 that expresses the T7 RNA polymerase following induction with heat (Tabor & Richardson, 1985). No transformants were successfully generated by this procedure and we presume that expression of the D6 open reading frame in a dut^-, ung^- mutant strain is lethal. The E. coli strain BL21 (DE3) carries the phage T7 RNA polymerase under the control of a lac UV promoter and the induction of T7 RNA polymerase activity with the subsequent expression of genes under the regulation of a T7 promoter can be achieved by the addition of isopropyl-β-thiogalactopyranoside (IPTG) to the growth medium. Synthesis of the D6 gene product was induced by adding IPTG to a final concentration of 0.4 mM to a 50 ml culture of mid log phase BL21 (DE3) harboring pXSUG-1. Following three hrs of induction the cells were harvested by centrifugation and the cell pellet was stored frozen at -70°C overnight. The cell pellet was thawed and resuspended in a solution of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 10% sucrose, 0.2 mg/ml lysozyme. After the cells had incubated on ice for 15 min Triton X-100 was added to 0.1%, and the cell debris was pelleted by centrifugation at 20,000 rpm in a Sorvall SS-34 rotor at 4°C for 20 mins. The induced 25 kDa protein was found as an insoluble high molecular weight aggregate in the cell pellet. The protein pellet was washed 3 times with lysis buffer plus 0.5% triton X-100 and then collected by centrifugation at 12,000 rpm in a microfuge. The pelleted protein was then solubilized in 8 M urea and allowed to incubate for 2 hours at room temperature. Insoluble material was removed from the solution by centrifugation at 12,000 x g for 10 min. The supernatant was then incubated at 4°C for 2 hrs and the concentration of urea was diluted to 4 M by the slow addition of 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 50 mM KCl, 0.01% NP-40 over 3 hours after which the solution was dialyzed for 16 hrs against 10 volumes of the dilution buffer with 4 changes over the 16 hr period. This solution was tested for uracil DNA glycosylase activity in the fluoresence loss assay and then concentrated to 1 ml by ultrafiltration using a Centricon 10 concentrator (Amicon) at 4°C. The refolded monomer size protein was then separated from the reaggregated fraction by gel filtration chromatography over a 15 ml column of Sephacryl S-300 (Pharmacia), 500 µl fractions were collected and tested for activity, the active fractions were pooled, concentrated by ultrafiltration and stored in 50% glycerol at -20°C.

C. Results

Infection of mammalian cells with poxviruses results in the induction of a series of enzymatic activities that are involved in nucleic acid metabolism. We have used a fluorimetric technique to assay crude lysates of SFV infected cells for the presence of a virally induced uracil DNA glycosylase activity. This assay has been described in detail

Figure VII-2. Fluoresence assays detect the induction of a uracil DNA glycosylase activity in the cytoplasm of SFV infected cells. All of the reactions were done at 37°C in the presence of 20 mM EDTA to minimize the influence of nucleases. (a) The fluoresence profile of a plasmid DNA substrate before heating (BH), and after heating (AH), after it has been incubated with a cytoplasmic extract made from cells that had been infected with SFV for 2 hours. The stable fluoresence over time suggests that little random nicking has occurred. (b) The after heat profiles of uracil containing plasmid DNA substrates after incubation with cytoplasmic extracts made from cells that had been infected with SFV for 2, 6, 12 hrs or were mock infected. The loss of fluoresence over time is indicative of the formation of AP sites or nicks in the DNA. (c) The fluoresence profile before heating (BH) and after heating (AH) of a uracil containing plasmid DNA after reaction with the 2 hr infected cell lysate in the presence of 10 mM uracil.


a
Control DNA (No uracil)
100
90
80
70
60
50
40
30
20
BH
AH
b
Induction of uracil DNA glycosylase activity
80
60
40
20
uninfected
12 hrs
6 hrs
2 hrs
c
Inhibition by 10 mM uracil
90
80
70
60
50
BH
AH
0
10
20
Fluoresence pH 12
Time (mins)

previously (Evans *et al.*, 1984, Morgan & Chlebek, 1989). Cytoplasmic extracts from cells that had been mock infected, or infected with SFV for increasing periods of time were assayed for the presence of uracil DNA glycosylase activity in the presence of 20 mM EDTA (Fig. VII-2). An induction of uracil-DNA glycosylase activity above the background of host cell activity can be detected as early as 2 hours after infection and the high level of activity was retained over the first 6 hours of the infection before declining in the late stages of the infection (Fig. VII-2b). The drastic decrease in fluorescence that is detected after heating the samples is indicative of the breakage of AP sites in the substrate DNA. The addition of 10 mM uracil (a specific inhibitor of most uracil DNA glycosylases) suppressed most but not all of the loss of fluorescence seen in the heated samples (Fig. VII-2c). We interpret this apparent suppression of breakage to be due to the inhibition of glycosylase activity. No uracil DNA glycosylase that has been characterized has been shown to have nuclease activity (Sakumi & Sekiguchi, 1990). However, the presence of AP endonucleases that have no requirement for divalent metal ions has been reported (reviewed in Doetsch & Cunningham, 1990). So it is possible that the creation of AP sites by the induced glycosylase activity allowed the DNA to be nicked. Suppression of the glycosylase activity prevents the creation of AP sites and thus protects the uracil containing DNA from nicking. The fluorescence of an identical DNA substrate that lacked any incorporated uracil remained unchanged following treatment with the lysate under the assay conditions (Fig. VII-2a). Thus we conclude that the alteration in fluorescence of our uracil containing substrate is due to the specific activity of a uracil DNA glycosylase that is induced early in the SFV replicative cycle prior to the initiation of viral DNA replication.

Oligonucleotide primers corresponding to the 5' and 3' ends of the coding region were designed and the D6 coding sequence was amplified by PCR. The amplified sequence was [^{32}P] labelled by the random priming technique and used as a hybridization probe for Northern blots of total RNA isolated from SFV infected cells. The D6 probe hybridizes most strongly to a transcript of approximately 700 nucleotides and also to a minor product

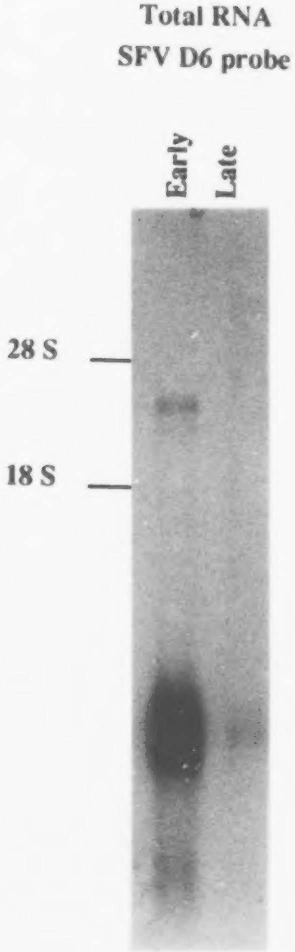


Figure VII-3. Expression of the SFV uracil DNA glycosylase gene. A Northern blot of total RNA isolated from SFV infected cells, and probed with a [^{32}P] labelled copy of the SFV D6 open reading frame. RNA was harvested from cells infected for 16 hrs in the presence of 100 µg/ml cycloheximide (Early RNA), or from cells that had been infected in the absence of drugs. In the latter case, the infection allowed to proceed in to the late stages (Late RNA). The position of the cellular 28S and 18S ribosomal RNAs are indicated on the left.

of higher molecular weight (Fig. VII-3, lane 1). The major transcript is detected at early times during the infection and is also present in RNA prepared from cells that had been infected with SFV in the presence of the protein synthesis inhibitor cycloheximide (Fig. VII-3, lane 1). The inhibition of protein synthesis prevents the uncoating of the poxviral genome, DNA replication, and the expression of late genes. However, the very efficient encapsidated transcription system is capable of producing transcripts from the early class of viral genes under these conditions. Some of the 700 nucleotide transcript is detected in RNA prepared at late stages of the infection (Fig. VII-3 lane 2) suggesting that it is a relatively stable message; however, the majority is clearly synthesized at early times.

In vitro translation of the D6 open reading frame RNA yielded a polypeptide that migrated in SDS polyacrylamide gel electrophoresis as a 25 kDa protein as predicted from the sequence of the gene (Fig. VII-4). The polypeptide was labelled very poorly with [^{35}S] methionine probably because only two methionine residues are coded for in the gene. A fluoresence assay for glycosylase activity demonstrated that the *in vitro* generated product was a bonafide uracil DNA glycosylase. The unprogrammed lysate demonstrated very low levels of glycosylase activity while the programmed lysate was very active in removing uracil from the plasmid substrate (Fig. VII-5). The slow time dependent rise in fluoresence demonstrated by the unheated DNA suggests that an endogenous AP endonuclease may be present in the lysate (Fig. VII-5).

The PCR amplified D6 open reading frame was self-ligated to generate concatemers and then cleaved with the restriction enzymes *Nde*I and *Bam*HI. The open reading frame fragment was then ligated into the expression vector pET-3a (Rosenberg *et al.*, 1987) to create pXSUG-1 with the SFV D6 open reading frame under the regulation of a phage T7 promoter (Fig. VII-6). In an initial attempt to verify that the gene product of the D6 open reading frame had uracil DNA glycosylase activity we transformed the E. coli strain CJ236 with the cloned gene in an effort to demonstrate complementation of the ung$^-$ phenotype. The cloned SFV D6 gene was stable in this strain while no source of T7 RNA polymerase

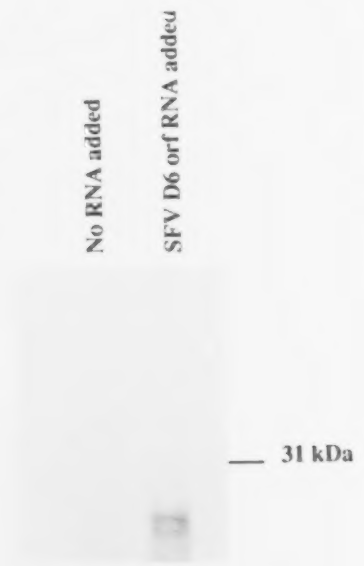


Figure VII-4. *In vitro* transcription and translation of the cloned SFV D6 ORF. The cloned SFV D6 gene was transcribed *in vitro* with T7 RNA polymerase and the purified RNA was translated in a rabbit reticulocyte lysate in the presence of [^{35}S] methionine. The translation products were fractionated on a 12% SDS-polyacrylamide gel. The molecular weight markers were visualized by Coomassie staining. The gel was dried and the labelled protein species were visualized by autoradiography. The control lane (No RNA) shows no labelled product, while the lysate programmed with D6 RNA shows a single polypeptide of 25 kDa.

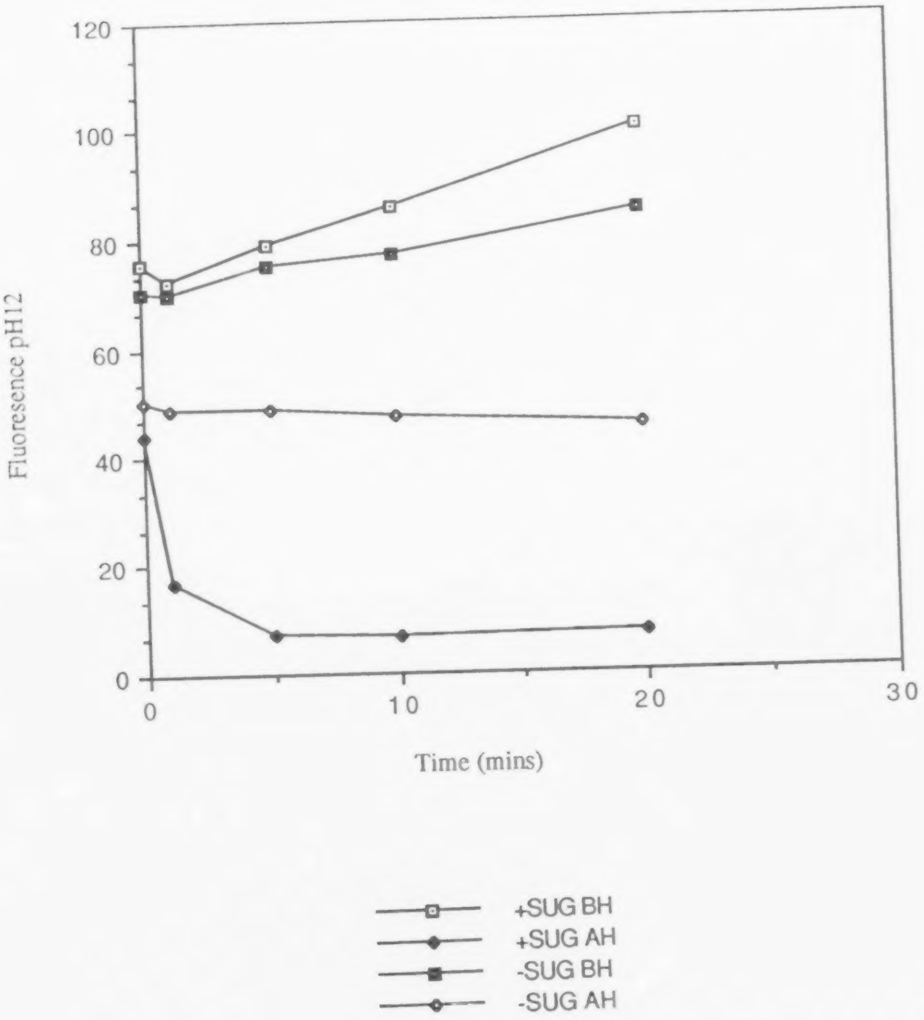


Figure VII-5. The *in vitro* translated D6 gene product displays uracil DNA glycosylase activity. Aliquots of the programmed (+ SUG) and unprogrammed (-SUG) reticulocyte lysates described in Fig VII-4 were assayed for uracil DNA glycosylase activity in a fluoresence loss assay. The very drastic and rapid decrease in fluoresence displayed by the uracil containing plasmid following treatment with the programmed reticulocyte lysate and heating (+SUG AH) compared with the relatively stable fluoresence of the same substrate DNA when treated with the unprogrammed lysate and heating, indicates that the SFV D6 gene product is a *bona fide* uracil DNA glycosylase.

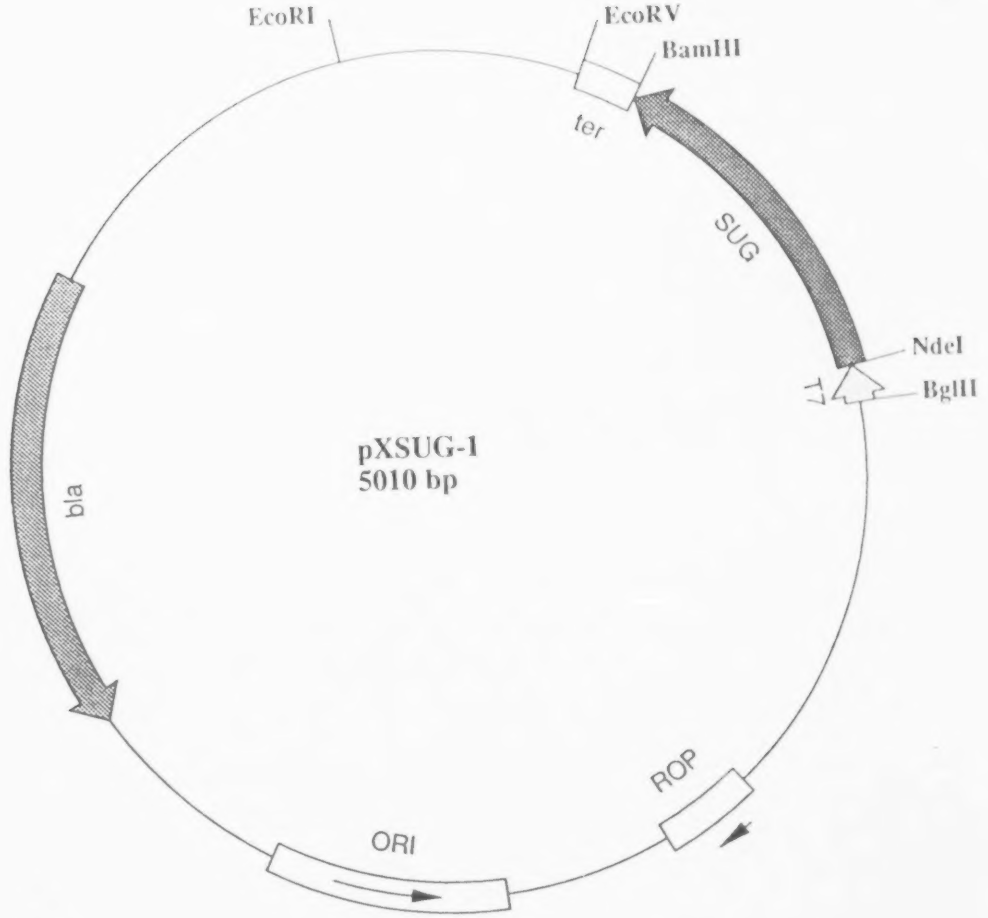

EcoRI
EcoRV
BamHI
ter
SUG
NdeI
T7
BglII
pXSUG-1
5010 bp
bla
ROP
ORI

Figure VII-6 The SFV D6 open reading frame was amplified by PCR using primers that generated a unique *Nde*I site within the translation initiation codon, and a unique *Bam*HI site immediately following the stop codon. This fragment designated SUG (SFV uracil glycosylase) was ligated into the an *NdeI*/ *Bam*HI cleaved expression vector pET-3a to generate pXSUG-1. The SFV D6 gene is flanked at its 5' end by a phage T7 promoter and associated ribosome binding site and at its 3' end by a phage T7 transcriptional termination sequence.

was present. T7 RNA polymerase was introduced into cells carrying the SFV D6 gene by transformation with the plasmid pGP2-1, which carries the phage T7 RNA polymerase gene under control of the lac UV promoter (Tabor & Richardson, 1985). We were unable to isolate cells that had been transformed by both plasmids (ampicillin^r and kanamycin^r) with any transformation protocol. Thus we presume that cotransformation events with the concomitant production of the D6 gene product are lethal to the dut$^-$, ung$^-$ strain. Since a demonstration of activity by complementation was not possible we introduced the expression vector pXSUG-1 into the E. coli strain BL21- (DE3). This strain carries the phage T7 RNA polymerase under the control of a lac UV promoter and thus expression of genes under the control of phage T7 promoters can be induced by the addition of IPTG to the growth medium. Induction of the T7 RNA polymerase by the addition of IPTG lead to the accumulation of a 25 kDa protein in the total cell lysates of cells carrying pXSUG-1 (Fig VII-7, lane 4). This species was present but was not so prevalent prior to induction (Fig. VII-7, lane 3). The 25 kDa band corresponds in size to the expected product from the 657 bp D6 open reading frame. The protein is synthesized in E. coli as a high molecular weight aggregate that can be quantitatively removed from the lysate by low speed centrifugation (Fig. VII-7, lane 6). E. coli contains a considerable amount of native uracil DNA glycosylase and it was not possible to detect an induction of activity over the host background in these cell lysates using the fluorimetric assay. Therefore we attempted to solubilize and refold the aggregated polypeptide in order to test its activity. Following solubilization in 8 M urea and slow dialysis at 4^{o}C it was possible to recover a significant amount of uracil DNA glycosylase activity (Fig. VII-8 a). This partially purified enzyme could be obtained as a fraction of the total protein from an S-300 gel filtration column (Fig. VII-7, lane 7) where it eluted with an apparent Mw of 26 kDa.

The SFV uracil DNA glycosylase has maximal activity in low salt conditions. Full activity was observed in reactions that contained up to 50 mM KCl but increasing the salt to 100 mM was detrimental to activity and a more significant loss of activity was observed at

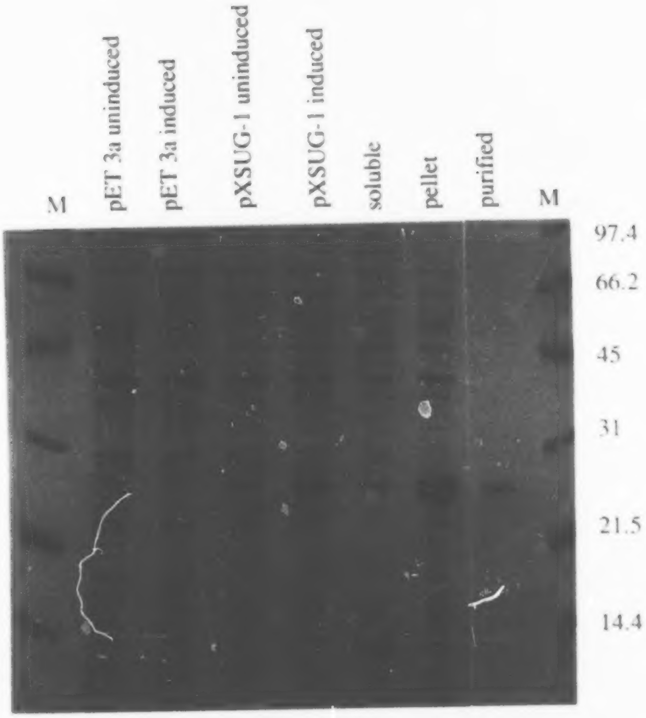
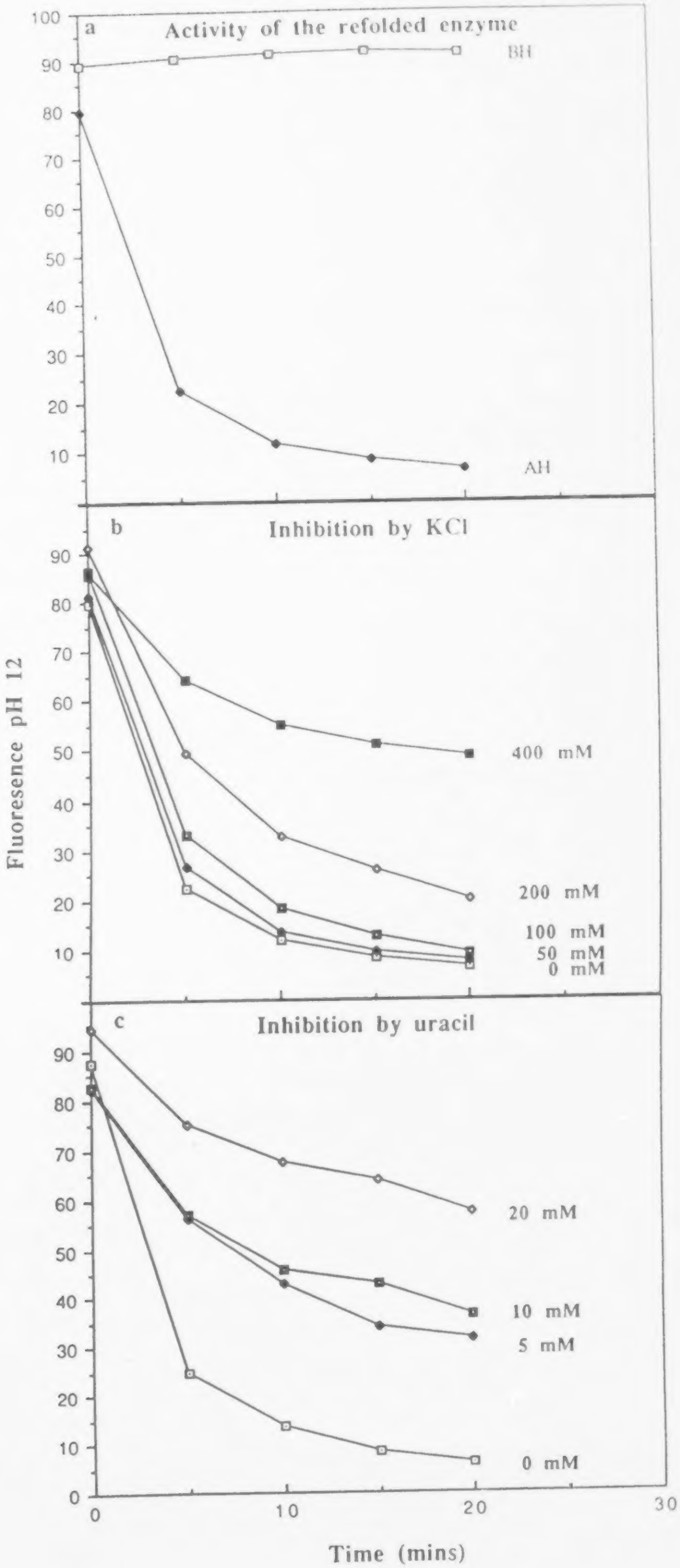

M
pET 3a uninduced
pET 3a induced
pXSUG-1 uninduced
pXSUG-1 induced
soluble
pellet
purified
M
97.4
66.2
45
31
21.5
14.4

Figure VII-7 Expression of the SFV D6 gene. Conditions for cell growth and induction are described in the text. Cell lysates harvested 3 hrs post-induction were fractionated on a 12% SDS-poiyacrylamide gel and the protein profiles were visualized by staining with Coomassie blue. M. molecular weight markers. Lane 1. total cell lysate from BL 21 (DE3) harbouring the parent vector pET-3a prior to induction with IPTG. Lane 2. total cell lysate from BL 21 (DE3) harbouring pET-3a made 3 hrs after the addition of 0.4 mM IPTG to the growth medium. Lane 3. total protein from BL 21 (DE3) carrying pXSUG-1 prior to induction. Lane 4. total protein from BL 21 (DE3) carrying pXSUG-1 3 hrs post induction. Lane 5 the soluble protein fraction from induced cells carrying pXSUG-1. Lane 6. insoluble protein from cells carrying pXSUG-1 post-induction. Lane 7. refolded uracil glycosylase isolated from S-300 chromatography.

Figure VII-8. SFV uracil DNA glycosylase expressed in E. coli can be solubilized in 8 M urea and successfully refolded. Fluoresence assays using a uracil containing plasmid DNA substrate, were performed using 5 µl of the refolded enzyme in a 50 µl reaction, (a) the fluoresence of the substrate DNA following reaction with the enzyme before heating to 95°C (BH) and after heating to 95°C for 5 mins (AH). (b) The activity of the SFV uracil DNA glycosylase as a function of KCl concentration. The fluoresence profile of uracil containing plasmid DNA is shown after it has been reacted with the refolded enzyme in the presence of the indicated concentration of KCl and heated to 95°C for 5 mins. (c) Product inhibition of the refolded SFV uracil DNA glycosylase by performing the reactions in the presence of the indicated concentrations of uracil. The fluoresence profiles of uracil containing plasmid DNA is shown after reaction with the refolded enzyme and heating to 95°C for 5 mins.


a
Activity of the refolded enzyme
BH
AH
100
90
80
70
60
50
40
30
20
10
b
Inhibition by KCl
400 mM
200 mM
100 mM
50 mM
0 mM
c
Inhibition by uracil
20 mM
10 mM
5 mM
0 mM
Fluoresence pH 12
0
10
20
30
Time (mins)

200 and 400 mM KCl (Fig. VII-8 b). The SFV uracil DNA glycosylase was product inhibited by increasing concentrations of uracil (Fig. VII-8 c). However some activity could be measured even in the presence of 20 mM uracil (Fig. VII-8 c). Thus in most respects the SFV enzyme appears to be very similar to its homologs in E. coli, yeast and all other organisms tested to date. It was of interest to determine if the SFV uracil glycosylase also had an AP endonuclease activity. This was tested by performing a modified fluoresence loss assay for uracil DNA glycosylase. Instead of heating the substrate DNA to denature the nicked species after treatment with the enzyme, the samples were added to 2 volumes of Me_2SO. After heating the substrate DNA to 55°C for 2 minutes all of the nicked DNA was denatured while DNA with AP sites remained intact. Treatment of uracil containing DNA with the partially purified SFV uracil DNA glycosylase resulted in no detectable nicking (Fig. VII-9). However, when the samples were heated to 95°C for 5 minutes a considerable loss of fluoresence was observed (Fig. VII-9). This suggests that the SFV enzyme, like all other known uracil DNA glycosylases, is capable of creating AP sites in uracil containing DNA, but cannot hydrolyze phosphodiester bonds in the plasmid backbone.

D. Discussion

We have identified and characterized a uracil DNA glycosylase encoded by the Shope fibroma virus. Poxviruses replicate in the host cell cytoplasm by virtue of an array of virally encoded DNA replicative enzymes (reviewed in Traktman, 1990) that are responsible not only for the mechanical aspects of viral DNA synthesis but also for the enhancement and maintenance of pools of DNA precursors. Several factors may contribute to the need for a virally encoded uracil DNA glycosylase. Extensive poxvirus DNA synthesis occurs in an apparently unregulated fashion supported by an expanded cellular pool of DNA precursors (Slabaugh *et al.*, 1991). Poxvirus infection specifically induces the the synthesis of DNA precursors by the expression of viral thymidine kinase (Hruby &

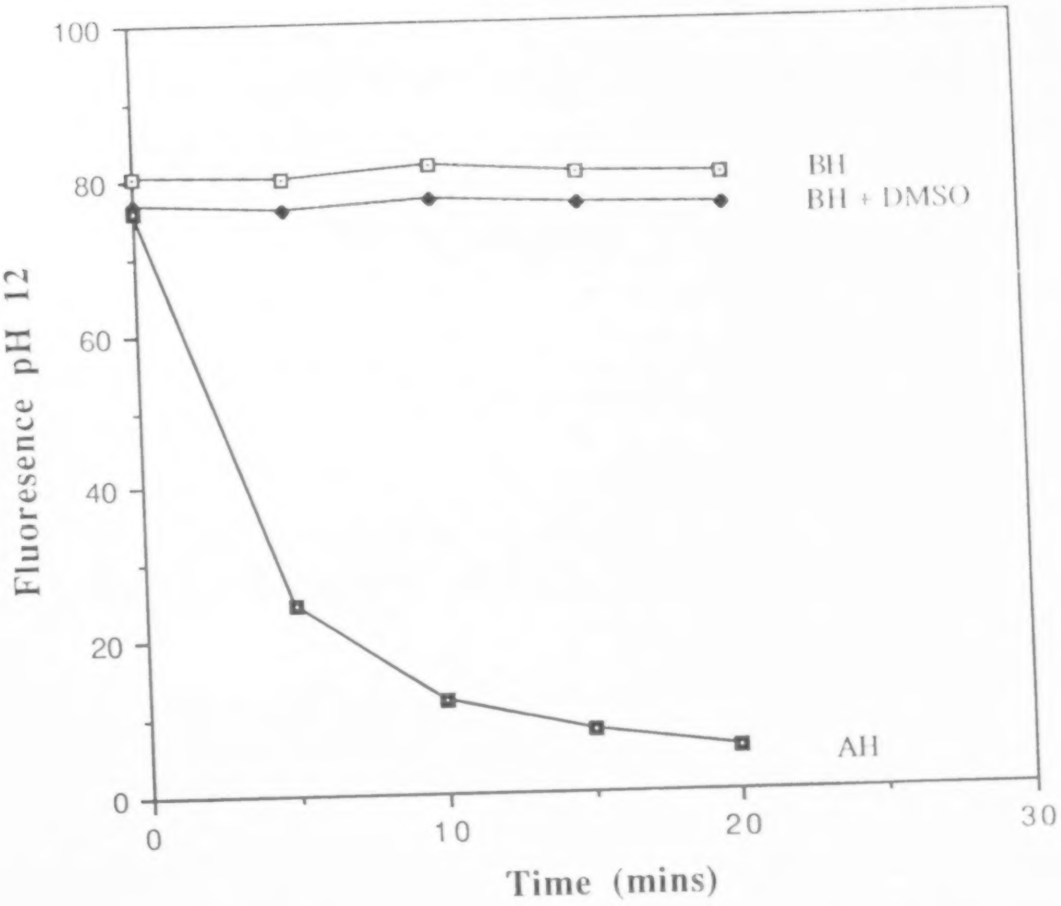


Figure VII-9. Fluoresence assay of the refolded SFV uracil DNA glycosylase using uracil containing plasmid DNA as substrate. Following treatment with the enzyme the fluoresence of the plasmid DNA was measured at pH 12 before heating (BH), after the addition of Me_2SO to denature nicked DNA (BH + Me_2SO), and then in the presence of Me_2SO after heating to 95°C for 5 mins (AH).

Ball, 1982), ribonucleotide reductase (Slabaugh *et al.*, 1984), and thymidylate synthase (Smith *et al.*, 1989). High intracellular nucleotide concentrations may influence the probability of misincorporating dUTP in place of dTTP. This might also explain the need for the putative poxvirus encoded dUTPase (McGeoch, 1990). The rapid shutoff of most host cell functions following poxvirus infection may limit the amount of the host dUTPase and uracil DNA glycosylase activities available and it has been reported that cellular levels of these enzymes are very low in quiescent cells (Cool & Sirover, 1989). Thus a viral homolog of each of these activities may be required to sustain high levels of accurate DNA replication. Poxvirus replication proceeds by the production of considerable amounts of single stranded DNA (reviewed by Traktman, 1990). Single stranded DNA is more susceptible to hydrolytic deamination of cytosine residues than is duplex DNA (Lindahl & Nyberg, 1974). Therefore, successful replication and minimization of mutation rates may require that poxviruses encode their own uracil DNA glycosylase along with other replicative enzymes.

Uracil DNA glycosylase activity is induced rapidly following infection, suggesting that the enzyme is required prior to and during DNA synthesis rather than as a post-replicative repair activity. Similar conclusions have been drawn with respect to the nuclear form of the enzyme in mammalian cells where an increase in uracil DNA glycosylase activity slightly precedes the peak of DNA synthesis in normally cycling or stimulated cells (Gupta & Sirover, 1981, Vollberg *et al.*, 1989).

The SFV induced uracil DNA glycosylase activity stands out prominently against the host cell background early during the infection but begins to decline as the infection proceeds into the late stages. The amount of activity that is detected in infected cell lysates is consistent with the abundant transcription of the SFV D6 gene at early times during the infection and its shut off at late times. The Shope fibroma virus uracil DNA glycosylase gene encodes a consensus signal for the termination of early viral transcripts (TTTTTAT) within its coding sequence and at the 3' end of the gene (Upton *et al.*, 1991 [submitted]).

The termination signal occurring at the 3' end of the gene is consistent with the size of the 700 bp transcript; however, the termination signal that occurs just 12 nucleotide pairs from the translation initiation site must be ignored by the transcriptional machinery. The mechanism of this antitermination phenomenon is not understood but it may involve the formation of secondary structure in the nascent RNA (Luo & Shuman, 1991) and could be a mechanism to down regulate the expression of this gene. The vaccinia virus homolog of the SFV uracil DNA glycosylase gene is also expressed early in the replicative cycle. However, the transcript encoding the vaccinia virus gene is not terminated at the 3' end of the gene (Lee-Chen & Niles, 1988). Rather a single transcript of 3.6 kbs is initiated from the uracil DNA glycosylase promoter and this transcript reads through the downstream gene, terminating within the coding sequence of the next open reading frame (Lee-Chen & Niles, 1988).

The fact that other DNA repair activities are not induced to a detectable level by SFV infection (Fisher *et al.*, 1991) suggests that repair of uracil incorporation in particular is very important. Another possibility that has not yet been investigated is that the SFV encoded enzyme serves multiple repair functions by creating AP sites from a variety of types of lesions. The uracil DNA glycosylase gene can be deleted from or disrupted in E. coli (Duncan, 1985), yeast (Burgers & Klein, 1986) and HSV (Mullaney *et al.*, 1989) indicating that the activity is not absolutely essential for growth and DNA replication. However, both the E. coli and yeast mutants display an increased sensitivity to mutagens (Duncan, 1985, Burgers & Klein, 1986) and the E. coli mutant displays an elevated spontaneous mutation rate (Duncan & Weiss, 1982). It has not yet been determined if the SFV uracil DNA glycosylase is essential for virus replication either in cultured cells or in animal tissue. The vaccinia virus homolog of the SFV uracil DNA glycosylase has been the target of intensive attempts at mutagenesis. Numerous attempts to inactivate the gene through the introduction of frame shift mutations and disruption of the gene with dominant selectable markers have failed (E. G. Niles, personal communication). While the inability to delete a gene does not necessarily constitute proof, it is at least circumstantial evidence that the gene serves an essential function . No reports of mutations in the uracil DNA glycosylase gene have been published; however, a conditional lethal mutation in the vaccinia virus IHD-W strain has been mapped to the uracil DNA glycosylase gene. (A. M. DeLange personal communication). This mutant virus designated ts4149 displays a marked defect in DNA synthesis at the nonpermissive temperature. Precisely how this DNA repair enzyme is involved in DNA synthesis is not clear. One hypothesis could be that the enzyme has some specific activity that has not been assayed for. A second explanation based upon the observation that the human uracil DNA glycosylase co-purifies with DNA polymerase alpha (Lee & Sirover, 1989), could be that the enzyme functions within a multiprotein DNA replication complex. The DNA repair activity may not be directly essential for DNA synthesis but the enzyme may have an important structural role in the replicative complex. Mutations in the gene product may disrupt the replicative complex leading to the observed defect in DNA replication. One way test this hypothesis would be to isolate extragenic suppressors of the defective DNA synthesis phenotype. Another strategy would be to demonstrate protein-protein interactions biochemically, via copurification and/or coimmunoprecipitation of the glycosylase with other enzymes known to be involved in DNA replication.

Now that the D6 gene has been cloned we should be able to address this question in several ways. Poxviruses are amenable to genetic manipulation by gene disruption or replacement by homologous recombination (Nakano *et al.*, 1982) and it should be possible to replace the native gene with *in vitro* generated mutants. Similarly when antibodies to the uracil DNA glycosylase become available it will be possible to attempt coimmunoprecipitations of the glycosylase and DNA polymerase. Finally the identification of extragenic suppressors in the DNA polymerase gene would provide strong evidence that these two enzymes interact with one another.

Our initial attempts to express the cloned SFV uracil DNA glycosylase in a dut$^-$, ung$^-$ E. coli strain were unsuccessful, likely due to the lethality of a dut$^-$ mutation in a uracil DNA glycosylase (Ung$^+$) background (elHajj *et al.*, 1988). One explanation for this lethality is that in the absence of dUTPase the cellular pool of dUTP increases with a consequent increase in the probability of dUTP misincorporation. The presence of uracil in DNA is not in itself lethal (Warner *et al.*, 1981) but the excessive excision repair, presumably catalyzed by the SFV gene product, may result in the introduction of double-strand breaks in DNA or might in some other way seriously compromise chromosomal DNA integrity. We have subsequently begun to use this lethality of SFV uracil DNA glycosylase expression as a genetic screen for interesting lethal or conditional lethal mutations in the cloned SFV gene.

We have been able to achieve efficient high level expression of the SFV uracil DNA glycosylase in Ung$^+$ E. coli. When synthesis of the enzyme is induced at 37^{o}C most of the gene product accumulates as an insoluble aggregate. A significant purification of the enzyme has been obtained in a single step by isolating and washing the insoluble aggregated protein. Successful solubilization and refolding of the protein has provided a significant amount of enzyme that has facilitated the biochemical characterization of the SFV uracil DNA glycosylase. Like all of its homologs in other organisms the SFV uracil DNA glycosylase displays no cofactor requirement and functions efficiently in the presence of high concentrations of EDTA. As well, the SFV enzyme displays no apparent phosphodiesterase activity. Thus it is capable of excising uracil to create an AP site but does not function to cleave the DNA backbone at this site. It can be argued that because the enzyme used in this study had to be refolded we may have renatured the glycosylase activity but failed to recover an endogenous nuclease activity. Expression of a soluble form of the enzyme or purification of the activity from SFV infected cells will be required to truly address this argument.

With respect to salt preference the viral enzyme appears to be similar to other uracil DNA glycosylases in that it functions well in up to 50 mM KCl. Increasing salt concentrations are inhibitory but no drastic decrease in activity is observed until the reactions are performed in the presence of 200 mM KCl. One aspect that does differ between the host and viral enzymes is the relative resistance of the viral enzyme to product inhibition; activity is observed even in the presence of 20 mM uracil, a product concentration that abrogates the host activity. One potentially confounding factor that has not been addressed by these studies is the effect of DNA methylation patterns on the activity of uracil DNA glycosylase. It is not known if poxviruses encode a DNA methylase; however, it is expected that the viral DNA would have a methylation pattern different from that of the plasmid DNA isolated from bacteria. Methylation patterns have been reported to affect the initial rate of uracil excision from DNA (Kuz'min *et al.*, 1988) however it seems unlikely that these differences would have an overall quantitative effect upon the assays utilized in these studies.

Now that we have identified a viral uracil DNA glycosylase clearly a question of interest is, how does excision repair proceed within the infected cell cytoplasms? Poxvirus infection induces the synthesis of at least three deoxyribonuclease activities (reviewed by Traktman, 1990). One of these viral enzymes may function as an AP endonuclease. Likewise, a host cell endonuclease might be recruited to create the nick required to initiate repair. Presumably the viral DNA polymerase is then involved in the repair synthesis, which is completed by the virus encoded DNA ligase. A reconstitution of this repair process should become possible with the expression of poxvirus DNA polymerase and the identification of the genes encoding the viral nucleases.

The simple isolation strategy and assay for the viral uracil DNA glycosylase coupled with a genetic screen for the identification of lethal or conditional lethal mutants should allow an extensive structure-function study to be performed with the subsequent identification of active site residues and the DNA binding domain.

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Chapter VIII

Conclusions and Recommendations

The major objective of the work presented in this thesis was to determine the mechanism by which the DNA replicative intermediates of poxviruses are resolved to yield mature progeny hairpin terminated genomes. While the complete answer to this question continues to elude us these investigations provide some partial answers, and some starting points for further investigations.

Overall I find the presentation of this thesis to be somewhat disappointing. This is principally because in spite of the many and varied tidbits of information that have been gathered together over the course of these studies I have been unable to congeal the data into a really coherent mechanism for telomere resolution. Perhaps more frustrating than disappointing has been the inability to apply "Oachims razor" to the problem and design the correct set of experiments to really dissect the problem and distinguish between possible mechanisms

Replicative Intermediate structure. The major conclusion that can be drawn from studies involving *in vitro* mutagenesis of a cloned version of the SFV replicative intermediate is that the inverted repeat structure of the viral concatemer junction plays an important part in the resolution mechanism distinct from the specific sequences that are required to initiate the event. An absolute requirement for inverted repeat symmetry demonstrated by the lethality of nonpalindromic insertions into the symmetry axis of the SFV replicative intermediate indicate that branch migration occurs on the pathway to forming the heteroduplex terminal hairpin structure. If this interpretation is correct then only two models for telomere resolution can be considered: (1) resolution of an extruded cruciform, and (2) protein mediated strand-exchange initiated within the TRT sequence. These investigations also identified a limit to the amount of sequence that could separate the two TRT regions without adversely affecting telomere resolution. While this is a useful piece of information, again it does not clearly distinguish between the two previously mentioned potential mechanisms for telomere resolution. It does; however, suggest communication between the TRT sequences through the intervening DNA rather than through a looping mechanism. Finally we were unable to detect any evidence of intermediates formed by protein mediated strand-exchange targeted to the TRT and branch migration.

TRT is a late promoter. Throughout the duration of these investigations my experimental model, the poxvirus, has visited one surprise after another upon me. One of the first and perhaps biggest was the observation that the TRT sequence, which is essential for telomere resolution, also functions as a strong promoter at late times in the viral replicative cycle. The TRT sequence behaves like a rather typical late promoter and based upon sequence is likely to be the strongest naturally occurring late promoter in the virus. I quite reasonably conclude from this that the viral RNA polymerase will be recruited to this sequence. This also implicates some part of the viral transcriptional complex in resolution, either directly through cleavage and recombination, or by providing helicase activity for unwinding the duplex of the replicative intermediate. A somewhat less profound finding of this study was that Orthopoxviruses and Leporipoxviruses will utilize each other's TRT sequences as both a *cis*-acting telomere resolution sequence and as a late promoter.

The transcriptional activity of the TRT can not be unlinked from the *cis*-acting resolution function. It can not yet be proven that the viral transcription complex plays a direct role in the resolution of telomere replicative intermediates. The inability to unlink these activities either mutationally or with transcription inhibitors suggests that activity from the transcription complex either by binding to and unwinding the TRT sequence, or binding and actively synthesizing RNA are essential features of the resolution mechanism. The observation that a limited number of viral late promoters other than the TRT will function in cis to resolve replicative intermediates further fuels this idea.

In vitro resolution of a cloned replicative intermediate structure by virally induced activities. The development of an *in vitro* system that can be used to study the mechanism of telomere resolution has been a goal that consumed an enormous amount of time and effort and in return has produced very meager results. The use of a crude lysate that was competent for the transcription of viral late genes has produced the only success to date in resolving replicative intermediates *in vitro*. Our crude extracts appear to have two activities that may be directly implicated in catalyzing resolution. The major activity, a Holliday junction resolvase, will correctly resolve cruciforms in a sequence nonspecific fashion. It is possible that this activity is the by product of a nuclease with loose specificity. However, it is just as likely that several different components act in conjunction to generate a secondary structure within the concatemer junction that can be cleaved by this viral resolvase. The viral RNA polymerase complex or some late transcription factor may be good candidates for proteins that induce secondary structure within the viral replicative intermediate. The second activity present in the crude lysate is an apparent TRT nicking enzyme. The generation of cleavages within the TRT sequence by a crude infected cell extract is very provocative and is consistent with the TRT being the site of strand-exchange initiation. On the other hand asymmetric cleavages in an inverted repeat could also be diagnostic of the resolution of a cruciform structure that had been extruded to the extent that the two copies of the TRT formed the base of the junction and became the target for the above mentioned Holliday junction resolvase. Finally, although this crude system has not yielded a great amount of information about either the mechanism or the biochemistry of the resolution event it appears to have good potential as a starting place for the isolation of particular activities that are involved in telomere resolution.

Homologous recombination. DNA replication and recombination are tightly linked events in the poxvirus replicative cycle. At the time that this project was undertaken it seemed a real possibility that homologous recombination or at least some of the activities involved in catalyzing homologous recombination might be playing a role in the resolution of the viral replicative intermediates. This is likely not the case since general recombination depends solely upon early gene products and DNA replication. However, it does set forth one of the major themes of this thesis and that is the concept of multiple functions performed by single viral enzymes or by complexes of viral enzymes that can "mix and match" individual subunits. Homologous recombination displays an absolute dependence on DNA synthesis catalyzed by the viral DNA polymerase. Whether the DNA polymerase is involved directly by a mechanism such as template switching, or simply elongates primers generated by strand invasion events has not been determined. No other viral enzyme has been shown to have any direct involvement in homologous recombination and the only characterized conditional lethal mutants that are defective in recombination are also defective in DNA synthesis at the nonpermissive temperature.

Poxvirus encoded uracil DNA glycosylase. In keeping with the above mentioned theme of multifunctional enzymes and enzyme complexes, I have overexpressed and characterized what first appeared to be generic DNA repair enzyme. The SFV encoded uracil DNA glycosylase does not seem to have any particularly unusual characteristics *in vitro*. It functions as a uracil glycosylase and has no intrinsic nuclease activity. An odd observation is that the gene encoding this enzyme is essential and cannot be inactivated in vaccinia virus. Furthermore, a conditional lethal mutation has been isolated in the vaccinia virus uracil glycosylase gene and this virus displays a profound defect in DNA synthesis at the nonpermissive temperature. One reasonable explanation for this behavior is that the uracil glycosylase functions in a replicative complex along with the DNA polymerase. Thus its DNA repair activity *per se* may not be essential to viral DNA synthesis but it may perform an essential structural role within the replicative complex. It would not be surprising, however, if this protein also has some activity that I have not assayed for or that is manifest only within the context of the intact replicative complex.

Prior to listing my suggestions for future work I wish to briefly discuss my feelings regarding some of our models for poxvirus telomere resolution. When I initially began thinking about this problem in 1986 I had the naive notion that a single specific viral enzyme was involved and that all I need do was apply the correct combination of purification techniques to isolate the activity. I now feel very strongly that a complex of viral proteins is involved in telomere resolution. It is likely that we are already familiar with some of the players in this game: the transcription complex, the mRNA capping enzyme, and maybe topoisomerase.

So how does resolution really happen ? I have significant doubts about the DNA looping model with subsequent strand-exchange initiated at the TRT and directed branch migration, for the following reasons. Distance between the TRT sequences should not have a significant influence on the looping event and small increases in distance should not influence the directed branch migration step, yet we observe a severe effect upon resolution with only very small increases in distance between the TRT sequences. DNA looping and TRT initiated strand-exchange topologically isolates the sequence domain between the inverted TRTs and each turn of helix that is required in branch migration requires a concomitant relaxation of one turn of helix, a very energetically demanding process. The TRT sequences in the native replicative intermediate are quite close together. Thus the energy required to loop the two domains together is very high, furthermore, the two TRTs can be brought to within 8 nucleotide pairs from one another without any decrease in resolution efficiency. There are two important considerations in the latter observation. (i) The energetic requirement for looping together sequences separated by only 8 base pairs approaches a very large number and (ii) No side of helix effect is observed at any distance between the TRT sequences. At long distances there is no doubt that the DNA is sufficiently flexible to allow proteins bound to different sides of the helix to interact, however, at such close proximity this seems highly unlikely. These particulars raise doubts in my mind about the significance of a looping model, but the virus may very well have found a mechanism to overcome the restraints that I have just described and make a looping mechanism energetically viable.

The cruciform extrusion model remains plausible mainly because I have not found any evidence that argues against it. No evidence exists to suggest that cruciform structures can be extruded in poxvirus infected cells, but the possibility that secondary structure formation is induced by the viral transcription complex cannot be discounted.

Have some possibilities been overlooked that could indicate yet another viable mechanism ? Something that I have wondered about is the topology of the replicating DNA in the virosomes. We tend to think about DNA and RNA polymerases as soluble enzymes that float around and jump on to available DNA templates, yet that is probably not the case in most organisms. More likely, the replicative complexes seem to be tethered at discrete sites by attachment to some part to the nuclear matrix and DNA seems to get dragged through the complex. Is the poxvirus replication complex tethered to some part of the virosome ? If it is, what are the implications for telomere resolution? Perhaps we could also ask whether the transcriptional complex is in some way tethered within the infected cells.

Recommendations for future experimentation. I think that the future of this problem lies in the reconstitution of the event *in vitro*. One good place to start may be the extract that I have described in this thesis, another good place to start may be by isolating the replicating virosomes under very mild conditions and then adding in defined substrates. This has some appeal because it does not demand that the replication/resolution activities become solubilized and dissociated prior to reconstitution. The major problem with monitoring resolution *in vitro* has been the detection of the event. Linear radiolabelled replicative intermediate structures may be the best substrate because these will avoid problems with the non-specific cleavage of Holliday junction structures or single stranded DNA. A more conventional approach that we have tinkered with in the past but that may be more successful now is the isolation of proteins that specifically bind to the TRT. Preliminary gel shift assays using the transcriptionally active extracts have been encouraging and even if this approach only pulls out transcription factors it may be leading on to the correct path. The genes for three late transcription factors have been identified so this might be a place to start. Biochemical complementation of cell extracts made at early times in infection with fractions of protein from extracts made at late times may also be a successful approach. Finally we know that only the central region of the virus encodes essential functions. It may be possible to hybrid select populations of viral late RNA and translate it, either in reticulocyte lysates, or preferably in oocytes (much more efficient), and assay for TRT binding proteins, helicases, or viral nucleases none of which have yet been cloned.

Appendix A

Depressed nucleoside transport in vaccinia virus infected cells. During the course of these studies, the membrane permeability and nucleoside transport capability of vaccinia virus infected HeLa cells was investigated. This study was prompted by the observation that at late times during infection, viral DNA synthesis is not accurately represented by the standard method of pulse labelling infected cells with [^{3}H]-thymidine (Traktman, 1990). 5 x 10^8 HeLa S3 cells in suspension culture were infected with vaccinia virus (WR) at a multiplicity of 15 pfu/cell, a T. K.$^-$ vaccinia virus strain at M.O.I.= 15 pfu/cell, or were mock infected. At 2 and 9 hrs post-infection aliquots of 6 x 10^7 cells were withdrawn from each culture, resuspended in 3 mls of 1 X sodium buffer (1 mM $CaCl_2$, 2.6 mM KCl, 1.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 138 mM NaCl, 8 mM Na_2HPO_4, 5 mM glucose) and divided into 8 sets of triplicate 100 µl samples. Set 1 was mixed with [^{3}H]-H_2O (6 x 10^3 cpm/µl), set 2 with [^{14}C]-sucrose (6 x 10^3 cpm/µl), and set 3 with [^{3}H]-polyethylene glycol (PEG) (6 x 10^3 cpm/µl). Each set was pulsed for 30 secs. The final 5 sets of triplicate samples were added, at times from 0-to-8 secs, to microfuge tubes that contained 800 µl of silicon/paraffin oil overlayed with 100 µl of [^{3}H]-thymidine pulse solution (2x sodium buffer, 0.15 µM [^{3}H]-thymidine, 9.85 µM thymidine), pulses were stopped by centrifuging the cells through the oil. Labelled nucleoside that remained in the upper aqueous phase was removed by aspiration, the upper part of the tube was then washed with H_2O to remove any remaining label and then the remaining oil was removed. The cell pellet was solubilized by the addition of 200 µl of 5% triton X-100, and counted to determine the amount of nucleoside taken up. The uptake is reported as pmol/µl cell water. [^{3}H]-H_2O was used to determine the total intracellular and extracellular water space of the cell pellet, and the membrane integrity was determined by permeability to labelled PEG and sucrose. The membranes of vaccinia virus infected HeLa cells remain intact as judged by impermeability to both PEG and sucrose for up to 9 hrs post-infection. This interpretation is made based upon the constant extracellular space determined with both sucrose and PEG (Table A-1). This finding is not in agreement with the permeability of infected cells reported by Carrasco & Esteban (1982); however, the experiments are not strictly comparable. A more interesting observation, is that infection with vaccinia WR appears to cause a depression of nucleoside uptake in infected HeLa cells (Fig. A-1). Comparison of the initial rate kinetics of all the data plotted in figure A-1 suggests that the the transporters on the surface of infected and uninfected cells are functioning at a similar rate. The depressed total uptake by infected cells at late times during infection (Fig A-1 ,WR 9h) can be explained by two possible mechanisms; (i) vaccinia virus infection has reduced the total number of functional transporters by direct inactivation of the transporter, or inhibition of host protein synthesis coupled with a normal degradation rate has reduced the number of transporters. (ii) A greatly expanded cellular pool of nucleosides and nucleotides caused by the induction of poxvirus enzymes results in a more rapid equilibration of labelled thymidine with the intracellular and extracellular space causing a decrease in the total uptake of labelled nucleosides. The turnover rate of cellular nucleoside transporters is not known; however, an expanded intracellular pool of DNA precursors is known to be induced by vaccinia virus infection (Slabaugh *et al.*, 1991); this might be acting to minimize the uptake of labelled nucleosides. Whatever the mechanism of transport inhibition, this may be at least part of the explanation for the inability to label nascent DNA at late times during poxvirus infection.

		2 hr post-infection	9 hr post-infection
	^{14}C-sucrose	31	27
mock	^{3}H-PEG	21	18
	^{14}C-sucrose	28.4	25.5
vaccinia WR	^{3}H-PEG	18.4	17
	^{14}C-sucrose	26.6	33
vaccinia TK^-	^{3}H-PEG	19.2	22

Table A-1 Vaccinia virus infection does not alter the permeability of cell membranes. The data are presented as the percent total cell water space occupied by sucrose or PEG 4000. The total water space was determined by the amount of [^3H] H_2O present in the cell pellet following centrifugation through oil. A significant increase in the space occupied by either sucrose or PEG is indicative of an increase in membrane permeability.

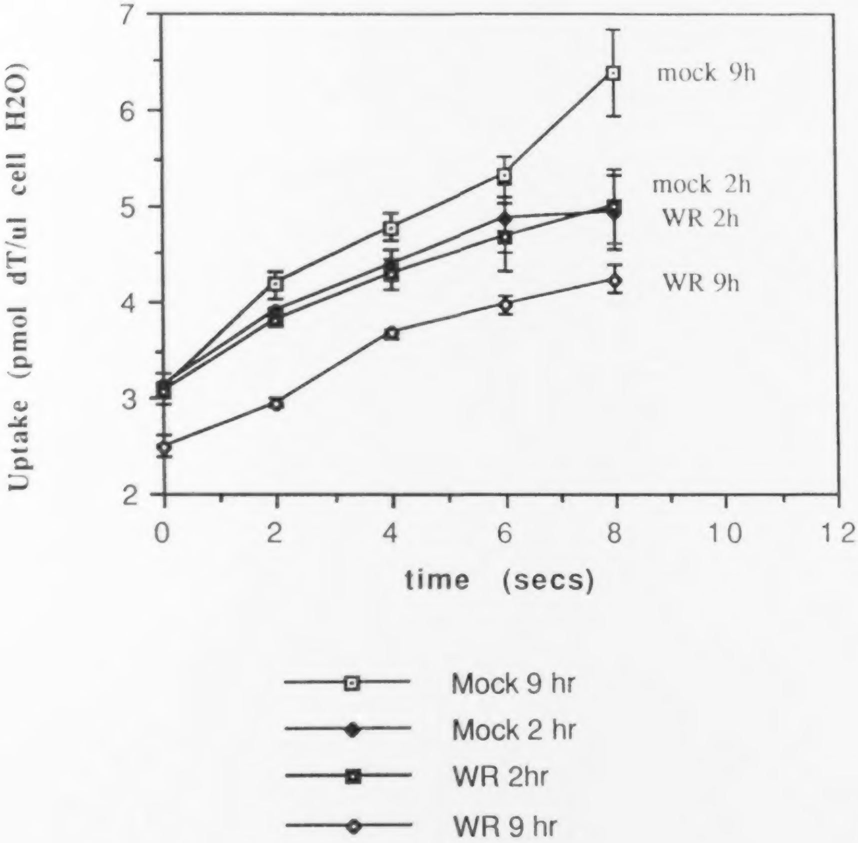

Figure A-1. Vaccinia virus infection results in a depression of cellular nucleoside transport. The transport assays were performed as described in the text. Data used to plot the curves is an average of triplicate samples. The standard deviations are indicated by vertical bars for each sample at each time point. The mock infected and WR infected cells display similar rates of transport at the early time period, this amount of transport is somewhat depressed from the more normal rate displayed by the 9 hr mock infected samples. We suspect that the slightly depressed transport is due to manipulation of the cells in the infection procedure.

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